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**Mechanisms Involved In Enhanced Protection Against Gram
Negative Bacterium *Francisella tularensis* Utilizing An FcR
Targeted Vaccine Platform**

by

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Submitted in partial fulfillment of the requirements for the degree

Doctor of Philosophy

Department of Biological Sciences

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I dedicate this work in loving memory of my father.

Table of Contents

Title	Page
Acknowledgement	iv
List of Figures	vi
List of Abbreviations	viii
Abstract	ix
Introduction	1
Aims of Study	13
Materials and Methods	14
Results	21
Discussion	53
References	67

List of Figures

Fig. 1 Administration of mAb-*iFt* immune complexes reverses the anti-inflammatory properties of LVS in mouse PECs *ex vivo*

Fig. 2 Immunization with mAb-*iFt* immune complexes increases the activation of PECs following LVS challenge

Fig. 3 *F. tularensis* LPS contributes to the anti-inflammatory properties of *F. tularensis* LVS during the early stages of infection

Fig. 4 Anti-inflammatory effect of *F. tularensis* LPS on mouse PECs is IFN- γ and IL-10 dependent

Fig. 5 Administration of mAb-*iFt* immune complexes reverses the anti-inflammatory properties of LVS in the lungs of immunized mice

Fig. 6 Fc γ R targeting drives polarization of mouse macrophages towards the AM1 phenotype at the early stages of LVS infection

Fig. 7 Immunization of mice with mAb-*iFt* immune complexes enhances the number of CD11c⁺ cells in the lungs of immunized mice following *F. tularensis* LVS challenge

Fig. 8 Increased frequency of activated CD11c⁺ dendritic cells in the lungs of mice immunized with mAb-*iFt* immune complexes and challenged with *F. tularensis* LVS

Fig. 9 Immunization of mice with mAb- *iFt* immune complexes increases the number of activated CD11c⁺ lung dendritic cells following *F. tularensis* LVS challenge

Fig. 10 Enhanced generation of effector memory CD4⁺ T cells in the spleen and lungs of mice immunized with mAb- *iFt* ICs, following *F. tularensis* LVS challenge

Fig. 11 Targeting of *iFt* to Fc γ R provides protection against *F. tularensis* LVS challenge in an IL-12-dependant fashion

Fig. 12 Generation of effector memory CD4⁺ T cells following targeting of *iFt* to Fc γ R, during *F. tularensis* LVS challenge, is IL-12-dependant

Fig. 13 Immunization of mice with mAb- *iFt* immune complexes increases the absolute numbers of effector memory CD4⁺ T cells, during *F.tularensis* LVS infection, in an IL-12-dependant fashion

Fig. 14 Increased generation of IFN- γ producing effector CD4⁺ T cells in mice immunized with mAb- *iFt* IC compared to mice immunized with *iFt* alone

List of Abbreviations

LVS	Live vaccine strain
<i>iFt</i>	inactivated <i>Francisella tularensis</i>
APC	Antigen presenting cell
PEC	Peritoneal exudate cell
SPC	Splenocyte
CFU	Colony forming unit
MOI	Multiplicity of infection
LPS	Lipopolysaccharide
TLR	Toll like receptor
IC	immune complex
mAb	monoclonal antibody
IFN- γ	Interferon gamma
IL-12	Interleukin 12
IL-10	Interleukin 10
TNF- α	Tumor necrosis factor alpha
PBS	Phosphate buffered saline
IgG	Immunoglobulin G
Fc γ R	Fc gamma receptor
MHC	Major Histocompatibility Complex
AM ₁	Classically activated macrophages
AM ₂	Alternatively activated macrophages
CD80	Cluster of differentiation 80
CD86	Cluster of differentiation 86
Th1	Type I T helper cells
Th2	Type II T helper cells

Abstract

Francisella tularensis is a gram-negative intracellular bacterium and the etiologic factor of a potentially fatal disease tularemia. Due to its high infectivity the Centers for Disease Control and Prevention has designated *F. tularensis* as a Category A biological agent. Nevertheless, the pathogenesis of *F. tularensis* is not fully understood and research has failed to develop an effective vaccine.

Production of pro-inflammatory cytokines by innate immune cells at the early stages of bacterial infection is important for host protection against the pathogen. Many intracellular bacteria, including *F. tularensis*, utilize the anti-inflammatory cytokine IL-10, to evade the host immune response. It is well established that IL-10 has the ability to inhibit robust antigen presentation by dendritic cells and macrophages, thus suppressing the generation of protective immunity.

In the current study, it is hypothesized that *F. tularensis* polarizes antigen presenting cells during the early stages of infection towards an anti-inflammatory status characterized by increased synthesis of IL-10, and decreased production of IL-12p70 and TNF- α in an IFN- γ -dependent fashion. In addition, *F. tularensis* drives an alternative activation of alveolar macrophages within the first 48 hours post-infection, thus allowing the bacterium to avoid protective immunity.

Furthermore, for the first time, this study demonstrates that targeting inactivated *F. tularensis* (iFt) to Fc γ receptors (Fc γ Rs) via intranasal immunization with mAb-iFt complexes reverses the anti-inflammatory effects of the bacterium on macrophages by

down-regulating production of IL-10 and mediates generation of *F. tularensis*-specific, IFN- γ secreting effector memory CD4⁺ T cells. Furthermore, targeting of iFt to Fc γ Rs enhances the classical activation of macrophages not only within the respiratory mucosa, but also, systemically, at the early stages of infection. These results provide important insight for further understanding the protective immune mechanisms generated when targeting immunogens to Fc γ receptors validating its potential as a universal vaccine platform against mucosal pathogens.

Introduction

Francisella Tularensis

Tularemia is a zoonotic infection characterized by onset of flu-like symptoms including fever, chills, headache and enlargement of lymph nodes. The causative agent of tularemia is *Francisella tularensis*, a gram-negative facultative intracellular bacterium that was first described in the literature in 1912 by McCoy and Chapin, as a bacterial pathogen that caused a plaque-like disease in rodents in Tulare County, California (McCoy & Chapin, 1912). The bacterium was initially named *Bacterium tularensis* but was later re-named *Francisella tularensis* to honor a researcher Edward Francis, who extensively studied the pathogen. *F. tularensis* can be transmitted through insect bites, infected carcasses, contaminated water, and inhalation of contaminated air, although inhalation of as little as 1-2 bacteria can lead to respiratory failure and death if left untreated (Ellis et al., 2002; Metzger et al., 2007; Sjostedt et al., 2007; Oyston et al., 2008). For this reason, the Centers for Disease Control and Prevention has designated *F. tularensis* as a Category A biological agent (Rotz et al., 2002). In fact, The World Health Organization (1970) predicted that an intentional spread of virulent *F. tularensis* over a metropolitan area would lead to 250,000 cases of infection including 19,000 lethal outcomes.

There are four subspecies of *F. tularensis*: *novicida*, *holarctica*, *mediasiatica* and the most virulent subspecies *tularensis* that has only been found in North America (Cole

et al., 2011). One of the properties of *F. tularensis* is its ability to survive and proliferate within phagocytic cells of the host. Upon the uptake into the host cell through phagocytosis, *F. tularensis* undergoes interaction with early and late endosomes after which a rupturing of the phagosomal membrane allows bacterium release into a cytosol (Santic et al., 2005; Chong et al., 2010; Geier et al., 2011). In the cytosol of non-activated cells the bacteria proliferates rapidly leading to apoptosis of the infected cells (Sjostedt, 2007). Thus, *F. tularensis* can evade a critical arm of the innate immune response by escaping from the phagolysosome pathway of myeloid cells. Another mechanism by which *F. tularensis* alters host cell immunity is increased synthesis of prostaglandin E₂ (PGE₂) (Woolard et al., 2007). PGE₂ is a potent regulator of the immune response by skewing it towards an overwhelming Th2 response and subsequent inhibition of T cell proliferation as opposed to generation of protective Th1 response.

Several studies reported that early innate immune responses, in particular secretion of inflammatory cytokines such as TNF- α , IFN- γ and IL-12, provide immediate control over *F. tularensis* replication (Leiby et al., 1992; Elkins et al., 2003; Duckett et al., 2005). *F. tularensis* infection, however, is characterized by the absence of early immune responses throughout the first 2-3 days after infection (Bosio et al., 2007; Rawool et al., 2008; Singh et al., 2013). This is believed to occur partly because of the low endotoxicity of *F. tularensis* lipopolysaccharide (LPS), which is structurally different from other Gram-negative bacterial LPS (Ancuta et al., 1996; Barker et al., 2006; Duenas et al., 2006; Hajjar et al., 2006). One of the differences is the lack of a phosphate at 4' position on lipid component A, another difference is that *F. tularensis* lipid A is tetra-

acylated and contains longer acyl chains of 16 to 18 carbons (Hajjar et al., 2006). These structural modifications interfere with the binding of LPS to a toll-like receptor 4-myeloid differentiation factor 2 (TLR4-MD2) complexes on host pattern recognition receptors, preventing the TLR4 oligomerization and activation of signaling, therefore, enabling *F. tularensis* to better evade the immune response.

***Francisella Tularensis* Vaccine**

Since no licensed vaccine for tularemia is currently available in the United States and around the world, there is a need for development of an effective vaccine.

Different approaches have been explored to identify routes of protection against lethal challenge with virulent type of *F. tularensis*, including whole bacteria and subunit vaccines. For instance, a vaccine developed by Foshay in 1940's, consisted of a whole *F. tularensis* bacteria killed through acetone extraction followed by phenol preservation. This vaccine was regarded as a safe formulation and was tested in animal models as well as administered to a large number of human volunteers. However, the results obtained from these studies did not show enough efficacies in protection against virulent strains of *F. tularensis* (Foshay et al., 1942). Furthermore, studies performed in the former Soviet Union using live attenuated strain of *F. tularensis* subspecies *holarctica* demonstrated more effectiveness in providing protection against bacterial challenge in comparison to Foshay vaccine. This vaccine was granted an investigational new drug status by the US Food and Drug Administration (FDA) and was named Live Vaccine Strain (LVS). Even though, the use of this vaccine generated quite promising results, the cause of its

attenuation is still unknown and consequently, LVS has not been approved by FDA for human administration (Conlan, 2004; Barry et al., 2009; Pechous et al., 2009; Schmitt et al., 2013). While attenuated in humans, LVS is highly virulent in rodents and therefore, is widely used in animal models to further understand the pathogenesis of tularemia.

Several studies have also investigated antigenic proteins on the bacterial surface such as LPS and outer membrane proteins (OMP) to assess their ability to trigger the protective immune response and to be further utilized for development of subunit vaccines against *F. tularensis* infection. Despite generating a protective antibody response to challenge with less virulent subspecies of *F. tularensis*, the subunit vaccines did not show significant efficacy to challenge with most virulent subspecies of the bacteria. Given that *F. tularensis* is an intracellular pathogen, the activation of both humoral and cell-mediated immunity might be required for stimulation of a sufficient immune response. Therefore, the search for safe and effective vaccine against infectious tularemia is still ongoing and various vaccine platforms are currently being evaluated.

Fcγ Receptor Targeting: Overview

Previous studies have shown that targeting antigens to Fc receptors (FcR) on antigen presenting cells (APCs) leads to enhancement of innate and adaptive immunity (Gosselin et al., 1992; Rawool et al., 2008; Gosselin et al., 2009; Bitsaktsis et al., 2012; Iglesias et al., 2012). Fcγ receptors are proteins on the surface of cells such as natural killer cells, macrophages, dendritic cells, neutrophils and mast cells. There are four classes of Fcγ receptors currently known in mice: FcγRI, FcγRII, FcγRIII and FcγRIV.

The first three classes of Fc γ receptors are highly homologous to the human Fc γ receptors. Structurally Fc γ receptors have similar extracellular domain, while the transmembrane and cytoplasmic domains are different (Gessner et al., 1998). Functionally, the Fc γ receptors differ by their affinities to their ligands and by signaling they initiate (Ravetch & Kinet, 1991; Nimmerjahn & Ravetch, 2007). The Fc γ receptors that stimulate cell activation, Fc γ RI, Fc γ RIII and Fc γ RIV, consist of an immunoglobulin binding α -chain and γ -chain containing signaling motif immunoreceptor tyrosine-based activation motif (ITAM). On the other hand, the only inhibitory Fc γ RIIB receptor contains α -chain and the immunoreceptor tyrosine-based inhibition motif (ITIM) (Daeron, 1997; Platzer et al., 2014). All Fc γ Rs bind with different affinities and specificities to various subclasses of immunoglobulin G (IgG). Yet, Fc γ RI is the only activating receptor that binds to IgGs, in particular IgG1 and IgG3 in humans, and IgG2b in mice, with significantly higher affinity compared to other Fc γ Rs (Gosselin et al., 2009). There are four IgG subclasses in humans (IgG1, IgG2, IgG3 and IgG4) and mice (IgG1, IgG2a, IgG2b and IgG3).

One of the characteristics of the activating Fc γ receptors is their ability to phagocytose antibody-antigen complexes and further facilitate presentation of processed antigenic peptides on MHC class I and MHC class II molecules to T cells (Regnault et al., 1999; Nimmerjahn & Ravetch, 2007). It is believed that these immune complexes (ICs) bind to Fc γ receptors via Fc region of antibodies leading to cross-linking of the receptors and subsequent uptake of the ICs. However, Igleasias et al. (2012) observed that the increase in the Fc γ R mediated antigen presentation required a physical linkage

between the mAb and antigen indicating that receptor cross-linking by itself is not sufficient for enhanced antigen presentation. The phagocytized antigens are then directed to the intracellular antigen presentation compartments (Baker et al., 2014; Platzer et al., 2014). Some studies suggest that the efficient intracellular transport of ICs is mediated by the neonatal Fc receptor (FcRn), an intracellular receptor expressed within antigen presenting cells that binds to IgG only under acidic conditions of endocytic vacuoles (Bitsaktsis et al., 2012; Baker et al., 2014; Guilliams et al., 2014).

It has been previously demonstrated that targeting inactivated *F. tularensis* (iFt) bacteria to the FcγRs in mice, via immunization with mAb-iFt immune complexes, resulted in: (1) enhanced uptake and presentation of the immunogen (iFt) by professional antigen presenting cells, (2) increased activation and maturation of dendritic cells, (3) enhanced *F. tularensis*-specific cytokine and antibody responses, and (4) increased protection against *F. tularensis* infection (Rawool et al., 2008; Iglesias et al., 2012). This protection was FcγR and FcRn dependent, as immunization of either FcγR or FcRn deficient mice as well as administration of F(ab')₂ mAb-iFt immune complexes abrogated protection (Rawool et al., 2008). In addition, it has been shown that targeting the pneumococcal surface protein A (PspA) to human FcγRI in FcγRI transgenic mouse model, also elicited enhanced protection against *Streptococcus pneumoniae* challenge (Bitsaktsis et al., 2012). Therefore, utilization of FcγR targeted approach provides a great potential for development of an effective vaccine strategy against tularemia.

Immune responses during *F. tularensis* infection

For many intracellular bacteria the induction of a robust innate immune response is a critical factor in host protection and bacterial clearance (Medzhitov & Janeway, 1997; Pashine et al., 2005; Kalinski, 2012). The induction of innate immunity is triggered upon recognition of bacterial components such as lipopolysaccharides, peptidoglycans, or bacterial DNA by cellular receptors in the early phases of infection and regulated by a number of pro-inflammatory cytokines including IL-12, TNF- α and IFN- γ and co-stimulatory molecules. Activation of the innate immune system, in turn, leads to initiation of more complex pathogen-specific adaptive immune responses responsible for bacterial elimination in the late phases of infection (Alfano & Poli, 2005; Lacy & Stow, 2011). However, as previously mentioned the *F. tularensis* infection lacks the early immune responses throughout the first 2-3 days after infection (Bosio et al., 2007; Rawool et al., 2008; Singh et al., 2013).

Interleukin-10 (IL-10), an anti-inflammatory cytokine secreted by many different cell populations including T cells, B cells, macrophages, dendritic cells and keratinocytes (Shibata et al., 1998; Pestka et al., 2004), inhibits pro-inflammatory cytokine synthesis and the antigen presenting ability of monocytes/macrophages and dendritic cells (Asadullah et al., 2003). A number of studies have demonstrated that many intracellular pathogens, such as *F. tularensis*, use IL-10 to evade the host immune defense especially in the initial stages of infection (Abrahamsohn & Coffman, 1996; Salek-Ardakani et al., 2002; Sing et al., 2002; Metzger et al., 2013).

The anti-inflammatory cytokine IL-10 is one of the key suppressors of host immune system that inhibits the antigen presentation ability of macrophages and dendritic cells mainly by down-regulating the expression of CD80, CD86 and MHC class II molecules, as well as the production of pro-inflammatory cytokines (Pestka et al., 2004). The ability of IL-10 to suppress innate, inflammatory responses against intracellular pathogens has been previously reported. For instance, lack of endogenous IL-10 production led to a decrease in number of *Trypanosoma cruzi* parasites in the blood of infected mice and increase in production of IFN- γ and nitric oxide (NO), which were reversed after administration of rIL-10 (Abrahamssohn et al., 1996). Similarly, lack of IL-10 resulted in higher resistance of mice to *Listeria monocytogenes*, characterized by lower bacterial burden, decreased tissue damage and increased pro-inflammatory cytokine response (Dai et al., 1997). Furthermore, the V antigen of *Yersinia pestis* caused suppression of the Th1 cytokine TNF- α in mouse and human macrophages by increasing IL-10 synthesis. In the same study, IL-10 deficient mice were highly resistant to the infection in comparison to wild type (Sing A. et al., 2002). In addition, viruses have also evolved mechanisms to escape the immune response by altering the Th1/Th2 balance. It has been shown that Epstein-Barr virus (EBV) utilizes the viral IL-10 (vIL-10), which is 84% identical in amino acid sequence to the human IL-10, to regulate the host immune response. Production of vIL-10 by EBV leads to inhibition of IFN- γ synthesis which is important for early regulation of EBV infection (Salek-Ardakani et al., 2002). Elevated levels of IL-10 which correlated with higher viral load in HIV infected individuals were observed by Brockman et al. (2009). However, inhibition of IL-10

pathway increased synthesis of pro-inflammatory cytokines IFN- γ and IL-2 reversing the suppression of virus-specific T cell functions that play a key role in controlling HIV infection (Brockman et al., 2009).

IL-10 has also been identified as a key regulator of immune response to *F. tularensis* infection. In particular, IL-10 mediates suppression of IL-17 production in *F. tularensis* LVS infected mice (Metzger et al., 2013). IL-17, in turn, is believed to play a protective role in the initiation of cellular immune response to *F. tularensis* LVS infection by induction of IL-12 and IFN- γ production (Lin et al., 2009). Several studies have demonstrated that blockade of TNF- α , IFN- γ and IL-12 signaling pathways decreases the survival rate in murine model during *F. tularensis* LVS infection (Leiby et al., 1992; Duckett et al., 2005). These cytokines activate production of nitric oxide in macrophages, which leads to the *F. tularensis* LVS bacterial killing by causing the double-stranded DNA breakage, bacterial Fe²⁺ release and depletion of antioxidant glutathione (Chakravorty et al., 2003; Lindgren et al., 2005).

F. tularensis predominantly infects antigen presenting cells such as macrophages and dendritic cells, the primary components of host defense mechanism. Therefore, activation of these cells plays an important role in controlling the replication of *F. tularensis*. Depending on the extracellular cytokine background, the activated macrophages are divided into two distinct groups, classically activated macrophages (AM1) associated with high levels of pro-inflammatory cytokines and alternatively activated macrophages (AM2) characterized by increased levels of anti-inflammatory cytokines, in particular IL-10 (Benoit et al., 2008). In addition to a distinct cytokine and

chemokine milieu, polarized macrophages portray different receptor expression (Mosser, 2003; Mantovani et al., 2004; Badylak et al., 2008; Benoit et al., 2008; Tomioka et al., 2012; Jaguin et al., 2013). Thus, CCR7, B7.1 (CD80), B7.2 (CD86) and MHCII have been identified as reliable surface markers that are up-regulated in classically activated macrophages. On the other hand, numerous studies have reported up-regulation of FIZZ-1 and Arg-1 in alternatively activated macrophages during infection, cancer and inflammatory conditions such as atherosclerosis (Raes et al., 2002; Benoit et al., 2008; Meghari et al., 2008; Ishii et al., 2009; Porta et al., 2009; Sanson et al., 2013). Classically activated macrophages are critical in control of replication of intracellular bacteria and therefore, required for host's defense against various infections (Katakura et al., 2004; Benoit et al., 2008; Shaughnessy et al., 2010). It has been previously proposed that one of the mechanisms that *F. tularensis* utilizes to subdue the protective immune response is its ability to induce the activation of alternatively activated macrophages to inhibit the protective M1 activation (Shirey et al., 2008).

Generation of Memory T cells

One of the main goals of vaccine strategy is the induction of a long-lasting protective memory. The properly activated antigen presenting cells play an important role in activation of pathogen-specific adaptive immunity (Beverley, 2002). The generation of a T-cell memory, in particular, is essential to long lasting protective immunity which consists of T helper lymphocytes or CD4⁺ and killer T lymphocytes or CD8⁺. In addition, there are two types of T memory cells, effector memory (T_{EM}) and central

memory (T_{CM}) cells that differ based on their functional responses as well as the expression of CD62L and CCR7 cell surface markers. Specifically, T_{CM} cells migrate to lymphoid tissues where they stimulate dendritic cells and induce a new generation of the effector T cells. On the other hand, T_{EM} cells are located largely in peripheral tissues where they activate a protective immune response against infectious pathogens including elevated production of inflammatory cytokines such as $IFN-\gamma$. Furthermore, T_{CM} cells express adhesion molecule CD62L and chemokine receptor CCR7 that are critical for T cell migration to lymph nodes, while T_{EM} cells are negative for both receptors (Gunn et al., 1998; Sallusto et al., 1999; Campbell & Butcher, 2000; Esser et al., 2003; McKinstry et al., 2010).

Polarization of T cells, regulated by antigen presenting cells via interaction with T cell receptor, is an important attribute of immune responses. There are three main subtypes of $CD4^+$ T cells that differ based on the secreted cytokine milieu – Th type 1 (Th-1), Th type 2 (Th-2) and Th type 17 (Th-17) cells (Sattler et al., 2009). Th-1 cells secrete pro-inflammatory cytokines such as $IFN-\gamma$ and $TNF-\beta$ that provide an effective cell-mediated response against intracellular pathogens. Th-2 cells secrete anti-inflammatory cytokines including IL-4 and IL-10 that trigger antibody-mediated response. Finally, Th-17 cells secrete cytokines such as IL-17, IL-17F and IL-22 that contribute to generation of protection against extracellular pathogens (Esser et al., 2002; Kaiko et al., 2007). T cell polarization largely depends on cytokines released by activated APCs and is triggered upon binding of these cytokines to their respective receptors on the surface of T cells. For instance, it has been previously shown that IL-12,

a heterodimeric cytokine produced by APCs in response to intracellular pathogens, is required for development of IFN- γ producing Th-1 cells. Moreover, the two cytokines act in an autocrine manner amplifying the Th-1 polarization and subsequently enhancing generation of an immune response (Macatonia et al., 1995; Schroder et al., 2004). Although cell mediated responses are critical to generation of long term immunity against intracellular infection, it has been shown previously that *F. tularensis* skews the T cell response from pro-inflammatory Th-1 towards anti-inflammatory Th-2 response, leading to inhibition of T cell activation (Woolard et al., 2007). Therefore, further understanding of the mechanisms utilized by *F. tularensis* to evade host immune responses will help for development of an effective vaccine against tularemia.

Aims of the study

1. To characterize the inflammatory responses in mice during early phase of *F. tularensis* infection

In the current study we hypothesized that *F. tularensis* polarizes antigen presenting cells (APCs) during the first 48 hours post-infection towards an anti-inflammatory status, characterized by IL-10 production, thus allowing the pathogen to avoid protective anti-bacterial innate immune responses.

2. To characterize the immune responses in mice upon delivery of immune complexes to Fc γ receptors during early phase of *F. tularensis* infection

We seek to determine whether targeting of mAb-*iFt* immune complexes to Fc γ Rs reverses the potential detrimental role of IL-10 during the early stages of *Francisella* infection.

3. To characterize the effect of Fc γ targeted mucosal vaccination on dendritic cell activation and memory CD4⁺ T cell formation *in vivo* during lethal challenge with *F. tularensis* LVS

It has been previously shown that targeting immune complexes to Fc γ receptors stimulates dendritic cell activation and maturation leading to enhanced *iFt* presentation to T cells *in vitro* (Iglesias et al. 2012). Nevertheless, questions remain regarding the *in vivo* impact of mAb-*iFt* immunization and whether DC activation and T cell priming also occur *in vivo* when utilizing the Fc γ R targeted vaccine strategy.

Materials and Methods

Mice and bacteria:

C57BL/6, IL-10 genetically deficient and IL-12p35 genetically deficient mice were purchased from Jackson Laboratories (Bar Harbor, Maine). All mice were housed at the Animal Research Facility at Seton Hall University. The mice were used at 6-10 weeks of age. All protocols were reviewed and approved by the Seton Hall University Ethics Committee utilizing NIH standards. Mouse anesthesia was performed via i.p. injections of a ketamine/xylazine cocktail, while mouse euthanasia was achieved via CO₂ administration followed by cervical dislocation.

F. tularensis LVS (ATCC 29684; American Type Culture Collection) was provided by K. Elkins (U.S. Food and Drug Administration, Bethesda, MD).

Antibodies:

Mouse IgG2a anti-*Ft*. LPS mAb used to generate mAb-i*Ft* immune complexes was purchased from Fitzgerald (Cat# 10-F02, clone#M023621, Acton, MA). The following flow cytometry antibodies were purchased from BD Biosciences (San Jose, California): F4/80 (PE), anti-CD3 (FITC), anti-CD4 (APC), anti-CD44 (PE), anti-CD62L (PercP Cy 5.5), anti-CD11b (FITC), anti-CD11c (APC), anti-IFN γ (PE), anti-DEC-205 (PercP Cy 5.5), anti-CCR7 (PE-Cy7), anti-CCR7 (PE-Cy5.5), MHC class II (APC), B7.1

and B7.2 (PercP). For neutralizing IL-12p35 *in vivo*, the rat IgG2a anti-mIL12 (clone: C18.2) and its isotype control were purchased from eBioscience (San Diego, CA).

Inactivation and Labeling of *F. tularensis*:

Inactivated *F. tularensis* LVS (*iFt*) was generated by growing *F. tularensis* LVS in Mueller Hinton broth (MHB) media (BD Biosciences) up to a density of 1×10^9 CFU/mL. The culture was then spun down at 22,000g for 20 minutes at 4°C, and washed 3 times with PBS, resuspended in 2% Paraformaldehyde (PFA) (Sigma) and incubated for 2 hours at room temperature on a rocker. Bacteria were then washed 3 more times with PBS and 1×10^9 organisms were plated on a chocolate agar plate (BD Biosciences) and incubated for 7 days at 37°C to confirm inactivation. The final concentration of *iFt* organisms was determined by OD at 610 nm.

mAb-*iFt* Immune Complex (IC) Generation:

To generate ICs, 1×10^9 *iFt* organisms were incubated at 4°C overnight on a rocker with 0 µg/mL or 1 µg/mL of mAb-*iFt* or anti-*iFt* F(ab')₂ in PBS. Following incubation, *iFt*, mAb-*iFt* or F(ab')₂-*iFt* preparations were washed once with PBS to remove any unbound antibody and administered to mice intranasally. Generation of ICs has been previously confirmed by ELISA and SDS-PAGE (Rawool et al., 2008; Iglesias et al., 2012).

Immunization and Challenge Studies:

C57BL/6, IL-10 deficient and IL-12p35 deficient mice were divided into three groups consisting of 5-6 mice/group, 6-10 weeks or 8-12 weeks of age. Each mouse was immunized on days 0 and 21 with 2×10^7 *iFt* organisms alone or in the form of mAb plus *iFt* ICs. On day 35 the mice were challenged with 10,000 CFU of live *F. tularensis* LVS. Following challenge survival was monitored twice-daily for 21-25 days. Death, due to the infection, was considered as the experimental end-point, although, in the occasions where animals were deemed to suffer (i.e. completely immobile, hunched backs, eyes shut), mice were sacrificed via CO₂ administration followed by cervical dislocation, per our approved animal protocol (Approval # CB1401). Exact CFU administered were also verified by culturing and counting the inoculum subsequent to challenge on chocolate agar plate.

Lung leukocyte isolation:

Lungs of immunized mice were harvested 24, 48 and 96 hours post-infection, perfused with cold 1x PBS containing a protease inhibitor cocktail, shredded into small pieces, and placed in digestion buffer containing RPMI (Life Technologies), 0.2mg/mL DNaseI (Sigma), 0.4mg/mL Collagenase D (Sigma), and 1M MgCl₂. After a 30 minute incubation at 37°C the digested tissue samples were forced through a cell strainer and the cell suspension obtained was washed and resuspended in RPMI containing 2% FBS. The cell suspension was then carefully layered on 5 mLs of Lympholyte M (Cedarlane Laboratories, Burlington, NC), and spun down at 15,000g for 30 minutes at room

temperature. Following centrifugation, the interface containing the majority of immune cells was obtained and added in RPMI with 2% FBS prior to enumeration. Identification and enumeration of alveolar macrophages was based on the expression of surface antigens F4/80 and CD11b. In case of DCs isolation, lung leukocytes were cultured in petri dishes containing complete medium at 2×10^6 cells/mL for 1 hour at 37°C. Identification and enumeration of DCs was based on the expression of CD11c and DEC-205. These markers are primarily expressed on mouse DCs (Kronin et al., 2000; Bonifaz et al., 2004). Nevertheless, a small percentage of alveolar macrophages (3-4%) are also positive for these markers (Higgins et al., 2008). Therefore, in order to further increase DC purity, lung white blood cells were cultured for 30 minutes at 37°C in petri dishes to remove (via adherence) the lung macrophages.

Peritoneal exudate cell (PEC) isolation:

PECs were harvested 48 hours post-infection, centrifuged in a refrigerated centrifuge 4,000g for 10 minutes. Following centrifugation, the interface containing PECs was resuspended in RPMI with 10% FBS, prior to enumeration. Identification and enumeration of PECs was based on the expression of surface antigens F4/80.

Neutralization of IL-12 *in vivo*:

Two doses of rat anti-murine anti-IL-12p35 mAb (C18.2) were administered intraperitoneally (i.p.) (500µg in 250µl of PBS) on days -1, 0 and 1 post-LVS infection.

This method has been proven to neutralize IL-12 *in vivo* for a minimum of five days post-administration of the IL-12p35 mAb (Meyts et al., 2006).

Flow cytometry:

Peritoneal exudate cells, lung parenchymal cells or splenocytes were obtained from immunized mice at different time-points post-*F. tularensis* LVS infection as described above. For cell surface marker staining, cells were washed with PBS-BSA-azide, resuspended in blocking buffer [PBS-BSA-azide plus 30 µg/mL of normal mouse IgG (Sigma)] and incubated on ice for 30 minutes. Cells were then washed with PBS-BSA-azide and fluorescently labeled antibodies to CD11b, CD11c, F4/80, DEC-205, MHC class II, B7.1, B7.2, CCR7, CD3, CD4, CD44, CD62L or their corresponding isotype controls were added. The cells were then incubated on ice for 30 minutes, washed, and then fixed with 2% paraformaldehyde (PFA). Cells were then analyzed by flow cytometry on an LSRII flow cytometer (BD Biosciences).

Intracellular cytokine staining:

Splenocytes (SPCs) from immunized mice were obtained 2 days post-LVS infection and cultured for 5 hours at 37°C in the absence or presence of LVS (1×10^3 CFUs per 2×10^5 SPCs). For a positive control, SPCs were cultured in complete media (RPMI, 2% FBS, L-glutamine), with 50ng/mL PMA (Sigma) and 500ng/mL ionomycin (Sigma). Cell media also contained protein transport inhibitor with 10 µg/mL of Brefeldin A (GolgiPlug – BD Biosciences). Following incubation, the cells were washed

with PBS-BSA-azide (containing the protein transport inhibitor), resuspended in blocking buffer, and stained with the cell surface marker antibodies for CD3, CD4, and CD44, and fixed with 2% PFA as described above.

After fixation, cells were washed, resuspended, and incubated in permeabilization buffer (BD Biosciences) for 15 minutes at 4°C to facilitate pore formation. Anti-mIFN- γ was diluted in permeabilization buffer and added to each cell sample. Cells were further incubated on ice for 30 minutes. Following incubation, cells were washed, resuspended in PBS-BSA-azide, and analyzed by flow cytometry on an LSRII flow cytometer (BD Biosciences). For intracellular cytokine staining of lung DCs, the latter cells were obtained from immunized mice, as previously described, 2 days post-LVS challenge. DCs were stained for the cell surface markers: DEC-205 and CD11, and intracellularly for IL-12, as described above. The results were analyzed by flow cytometry on an LSRII flow cytometer (BD Biosciences).

Cytokine measurements:

C57BL/6 mice were immunized intranasally (i.n) with PBS, i*Ft* (1×10^9 CFUs), or mAb-i*Ft*, boosted on day 21 and challenged on day 35 with either 10,000 CFUs or 20,000 CFUs of *F. tularensis* LVS. After 48 hours post-infection peritoneal cells or lung DCs were obtained from all groups and cultured for 24 or 48 hours with either *F. tularensis* LVS (1:10 and 1:100 MOI or 1×10^3 CFUs/well), *F.tularensis* LPS (kindly provided by Dr. Timothy Sellati – Albany Medical College, Albany, NY) or *E. coli*-LPS (Sigma) at 1

ng/mL, 10 ng/mL and 20 ng/mL, or recombinant IFN- γ (Invitrogen) at 100 U/mL. Supernatants were collected at designated time points and the levels of IL-12p70, TNF- α and IL-10 cytokines were measured using BD Biosciences Cytometric Bead Array (CBA) following vendor instructions.

In a separate experiment, lung tissue (left lobe) was harvested from immunized mice and homogenized (Omni Homogenizer, Omni International, GA). Homogenates were then spun down at 15,000g for 30 minutes at room temperature to remove tissue debris and cytokine levels were detected by using the IL-6, IL-10, TNF- α and IFN- γ ELISA kits by following vendor instructions (Biolegend).

Statistical Analysis:

The Log-Rank (Mantel-Cox) test was used for survival curves. Statistical differences among the groups were analyzed using a one-way analysis of variance (ANOVA) or the unpaired, one-tailed student t-test. GraphPad Prism 4 provided the software for the statistical analysis (San Diego, CA).

Results

Administration of mAb-*iFt* immune complexes (ICs) reverses the anti-inflammatory properties of *F. tularensis* LVS *ex vivo* and increases the activation of mouse peritoneal exudate cells (PECs)

One of the critical immune responses to bacterial infection is the synthesis and release of pro-inflammatory cytokines by innate immune cells during the early stages of infection (Medzhitov & Janeway, 1997; Pashine et al., 2005; Kalinski, 2012). Given the ability of *F. tularensis* to evade the immune system by favoring the short-term secretion of anti-inflammatory cytokines (Bosio, 2011; Metzger et al., 2013), it was of interest to investigate the cytokine levels produced by PECs at the early stages of infection. Therefore, production levels of inflammatory cytokines IL-12p70 and TNF- α as well as the anti-inflammatory cytokine IL-10 were analyzed using PECs from immunized and subsequently challenged mice. On day 35 post-immunization mice were challenged with 10,000 CFU of live *F. tularensis* LVS and cells were isolated two days post-infection. PECs were further stimulated with *F. tularensis* LVS *ex vivo* (at 1:10 and 1:100 MOI) for 24 hours and the cytokine levels in the supernatant were measured by the BD Biosciences Cytometric Bead Array (CBA). The levels of IL-12p70 (Fig.1A) and TNF- α (Fig. 1B) in supernatants from PECs of mAb-*iFt* immunized mice were significantly higher (two to three fold) in comparison to mice immunized with *iFt* alone. By contrast, the levels of IL-10 production were 2-fold lower in the mAb-*iFt* compared to mice immunized with *iFt* alone (Fig. 1C) independent of the MOI tested.

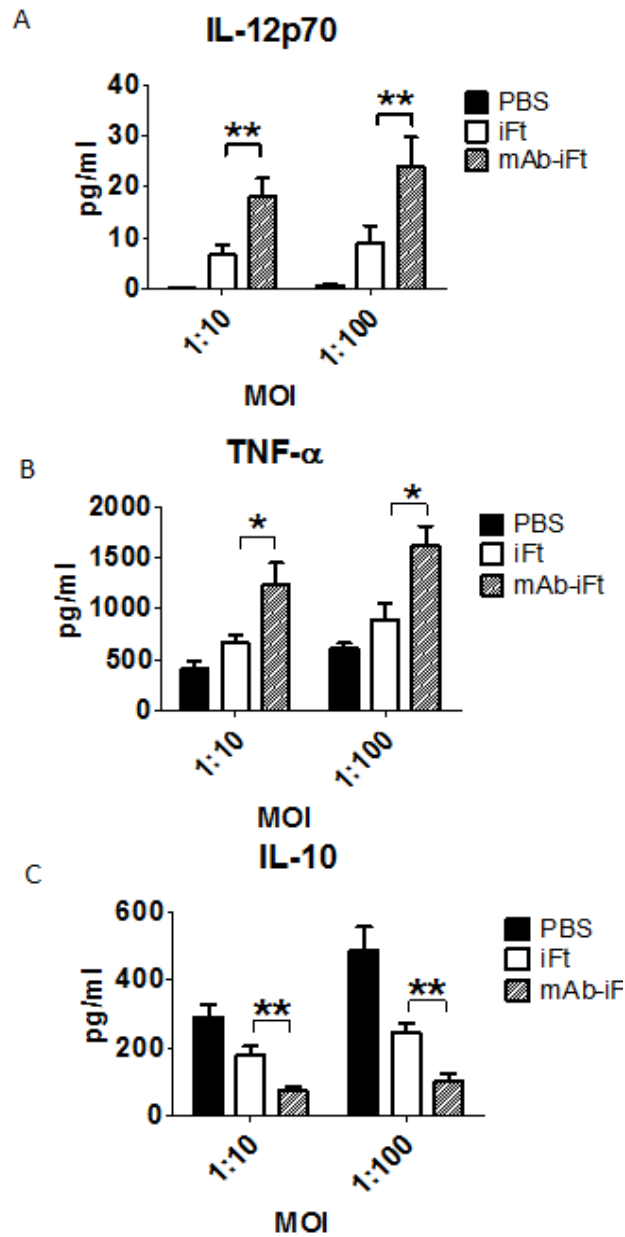


Fig.1 Administration of mAb-*iFt* immune complexes reverses the anti-inflammatory properties of LVS in mouse PECs *ex vivo*

C57BL/6 mice (n=6 for each group) were immunized i.n. with PBS, *iFt* (2×10^7 CFUs), or mAb-*iFt*, boosted on day 21 and challenged on day 35 with 10,000 CFUs of *Ft* LVS. After 48 hours after *F. tularensis* LVS challenge, the PECs of immunized mice were harvested and cultured in the presence or absence of *F. tularensis* LVS at 1:10 and 1:100 MOI for 24 hrs. The cytokine production was measured as previously described. Results shown are mean \pm SD from three independent experiments determined by one-way ANOVA (* P-value < 0.1; ** P-value < 0.05).

IL-10 is known to decrease the cell surface expression of MHC class II and co-stimulatory molecules CD80 and CD86 on murine macrophages (Ding et al., 1993). However, one of the mechanisms by which immune complexes trigger immune responses is via T cell activation that requires robust antigen presentation by activated antigen presenting cells and increased expression of co-stimulatory molecules. Previous experiments have shown that co-culturing of *F.tularensis*- specific T cell hybridoma with mouse PECs in the presence of mAb-*iFt* noticeably increased *F.tularensis*-specific T cell responses compared to using the *iFt* immunogen alone (Iglesias et al., 2012). Therefore, the hypothesis was that targeting of immunogen to Fc γ receptors (Fc γ Rs) increases systemically the activation status of antigen presenting cells following *F. tularensis* LVS challenge. Thus, the expression of MHC class II and co-stimulatory molecules CD80 and CD86 were measured on PECs from immunized mice post-infection.

Peritoneal cells were obtained from immunized mice two days after *F. tularensis* LVS challenge as described in Materials and Methods, and the expression of the murine macrophage cell-surface marker F4/80, the co-stimulatory molecules CD80 and CD86, as well as MHC class II was determined by flow cytometry. Although the number of cells expressing the F4/80 cell surface marker was similar between the *iFt* and mAb-*iFt* immunized mice, the number and frequency of cells expressing both MHC class II and CD80/CD86 molecules was significantly increased in the mAb-*iFt* group (Fig. 2). This enhancement in surface marker expression upon immunization with mAb-*iFt* correlates with the increased presentation of *iFt* in the presence of mAb-*iFt* in vitro (Iglesias et al., 2012).

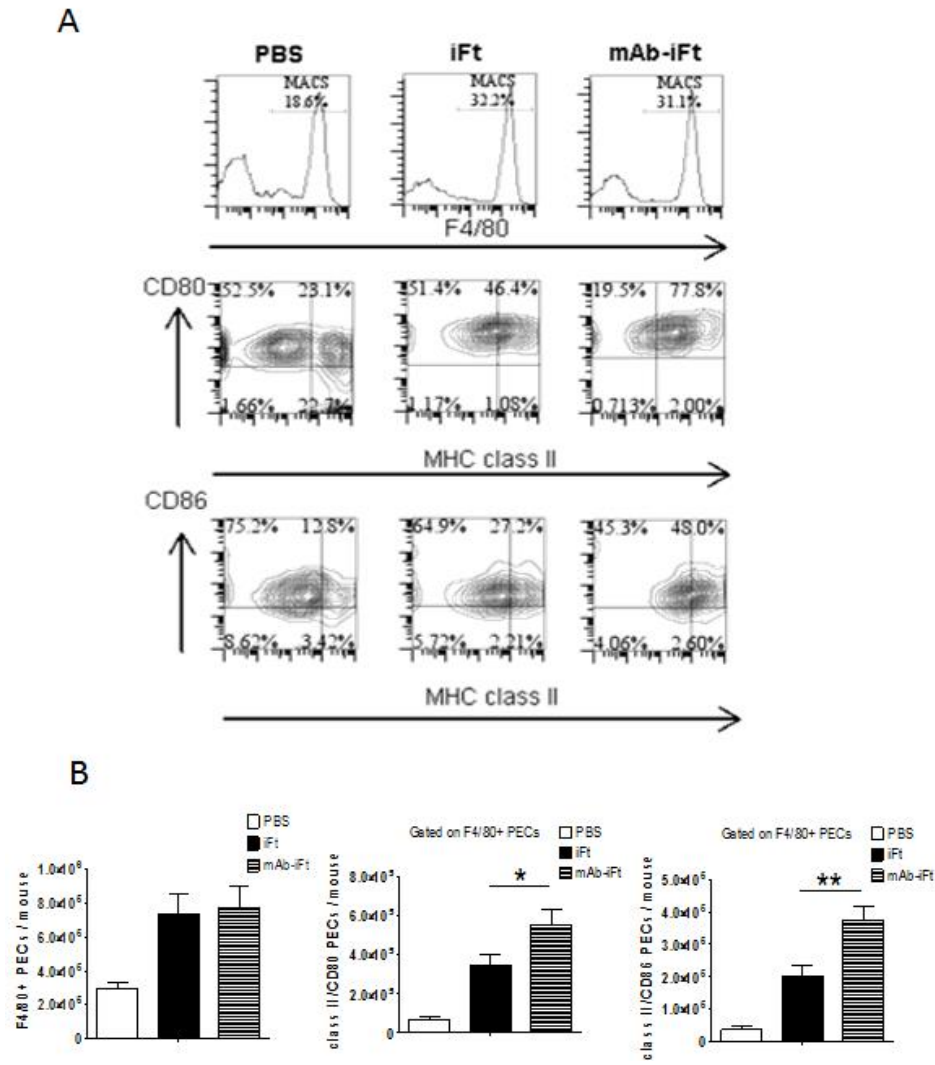


Fig. 2: Immunization with mAb-*iFt* immune complexes increases the activation of PECs following LVS challenge

C57BL/6 mice (n=6 for each group) were immunized i.n. with PBS, *iFt* (2×10^7 CFUs), or mAb-*iFt*, boosted on day 21 and challenged on day 35 with 10,000 CFUs of *iFt* LVS. On day 2 post-infection the peritoneal exudate cells of immunized mice were harvested and the expression of F4/80, MHC class II, B7.1 (CD80), and B7.2 (CD86) were analyzed by flow cytometry. Results shown are mean \pm SD from three independent experiments determined by one-way ANOVA and Student's t-test (* P-value < 0.1; ** P-value < 0.05).

These results demonstrate for the first time that intranasal immunization with mAb-iFt favors a pro-inflammatory cytokine profile secreted by murine macrophages as depicted by an increase of IL-12p70 and TNF- α production and inhibition of IL-10. Moreover, it triggers activation of peritoneal macrophages during *F. tularensis* infection, indicating the induction of a systemic response *in vivo*.

***F. tularensis* LPS contributes to the anti-inflammatory properties of *F. tularensis* LVS in an IFN- γ and IL-10 dependent manner**

It is well established that *F. tularensis* LPS is structurally different from other intracellular bacteria and elicits a subdued inflammatory response in the initial stages of infection, which otherwise are critical in controlling bacterial burden (Ancuta et al., 1996; Barker et al., 2006; Duenas et al., 2006; Hajjar et al., 2006). To investigate the effect *F. tularensis* LPS on the cytokine profile produced by PECs, the levels of IL-12p70, TNF- α and IL-10 were measured in the supernatants of PECs from naïve C57BL/6 mice cultured with various concentrations of *F. tularensis* LPS. As expected, incubation with *F. tularensis* LPS at different concentrations triggered significantly lower levels of IL-12p70 (Fig. 3A) and TNF- α (Fig. 3B) secretion compared to *E. coli* LPS (positive control). In contrast, IL-10 production was elevated in the presence of *F. tularensis* LPS (Fig. 3C). This observation not only confirms the low endotoxic activity of *F. tularensis* LPS but also indicates that it down-regulates Th-1 cell mediated inflammatory responses via up-regulation of IL-10 and down-regulation of IL-12p70 and TNF- α .

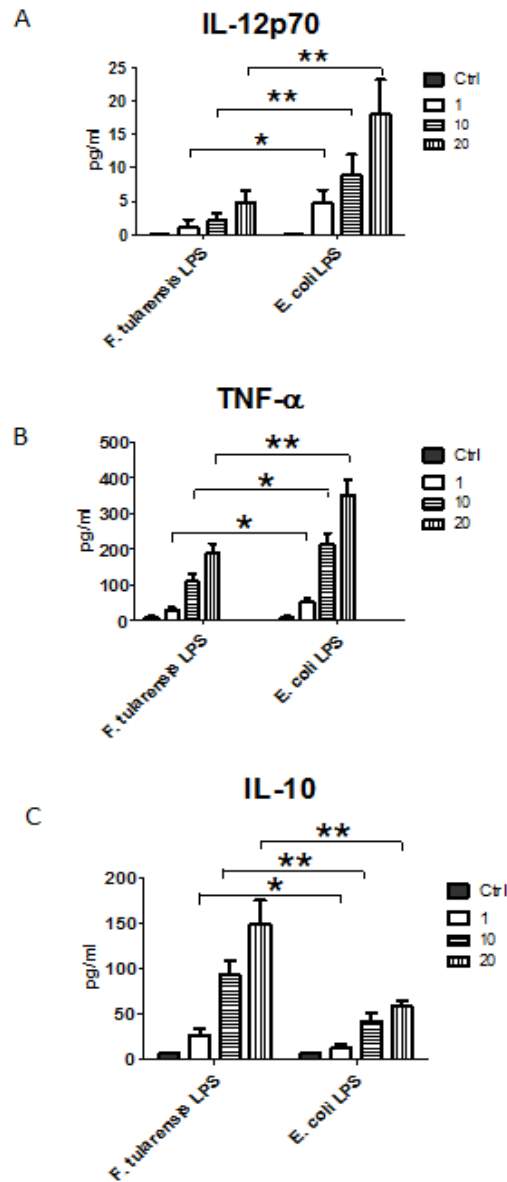


Fig. 3 *F. tularensis* LPS contributes to the anti-inflammatory properties of *F. tularensis* LVS during the early stages of infection

PECs from C57BL/6 mice (n=5 for each group) were obtained and cultured in a 96-well plate at 2×10^5 cells/well in the presence or absence of either *Ft*-LPS or *E. coli*-LPS at 1 ng/mL, 10 ng/mL and 20 ng/mL. Cells incubated with PBS were used as a control. Levels of IL-12p70, TNF-α and IL-10 were measured using BD Biosciences Cytometric Bead Array (CBA) following vendor instructions. Results shown are mean \pm SD from three independent experiments determined by one-way ANOVA (* P-value < 0.1; ** P-value < 0.05).

Several studies have shown that IL-10 negatively regulates synthesis of IFN- γ as well as synthesis of other pro-inflammatory cytokines in monocytes and macrophages (Donnelly et al., 1995; Brustoski et al., 2005; Cope et al., 2011). Due to the increased levels of IL-10 synthesis after incubation of PECs with *F. tularensis* LPS it was of interest to examine whether this up-regulation correlates with the decrease in TNF- α and IL-12p70 secretion. To investigate this, PECs from C57BL/6 wild-type and IL-10 genetically deficient mice were incubated with *F. tularensis* LPS or *E. coli* LPS. As previously observed, in the absence of endogenous IFN- γ , *F. tularensis* LPS portrayed anti-inflammatory properties via up-regulation of IL-10 production and decrease in the synthesis of IL-12p70 and TNF- α by the PECs. Interestingly, these results were reversed by the addition of exogenous IFN- γ (Fig. 4A-4C). Lack of endogenous IL-10, in turn, resulted in an elevated synthesis of IL-12p70 (Fig.4D) and TNF- α (Fig. 4E). These results indicate that increased levels of IL-10 synthesis is one of the mechanisms responsible for suppressing the protective pro-inflammatory cytokines such as TNF- α and IL-12p70 leading to an overall suppression of the Th-1 response and reduced IFN- γ levels in the early stages of *F. tularensis* infection. In fact, the importance of IFN- γ during *F. tularensis* infection was reported by Rawool and colleagues (Rawool et al., 2008), who observed a significant drop in survival rate in the mAb-iFt immunized IFN- γ ^{-/-} mice compared to the wild type control.

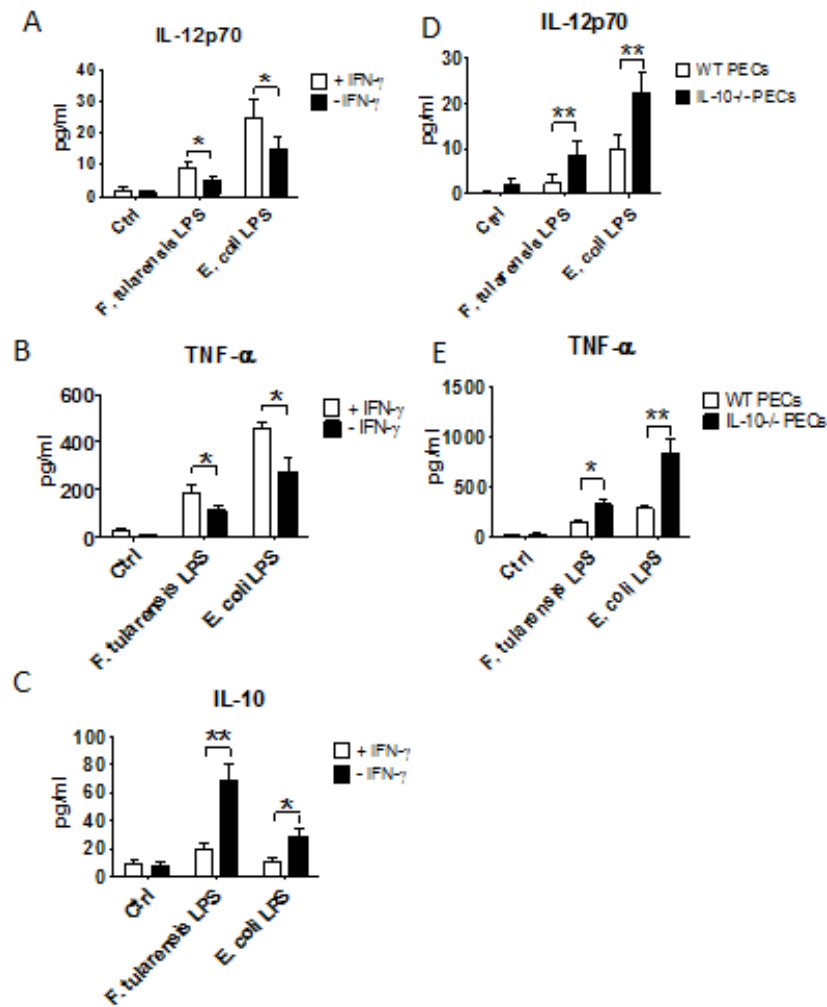


Fig. 4 Anti-inflammatory effect of *F. tularensis* LPS on mouse PECs is IFN- γ and IL-10 dependent

PECs from naïve and IL-10 genetically deficient C57BL/6 mice (n=5 for each group) were obtained and resuspended in cell culture media. PECs were cultured in a 96-well plate at 2×10^5 cells/well with either *Ft*-LPS or *E. coli*-LPS at 1 ng/mL in the presence or absence of recombinant IFN- γ at 100 U/ml. Cells cultured with PBS were used as a control. The cytokine production was measured using BD Biosciences Cytometric Bead Array (CBA) following vendor instructions. Results shown are mean \pm SD from three independent experiments determined by one-way ANOVA and Student's t-test (* P-value < 0.1; ** P-value < 0.05).

Administration of mAb-*iFt* immune complexes reverses the anti-inflammatory properties of *F. tularensis* LVS in the lungs of immunized mice

The balance and kinetics of pro- and anti-inflammatory cytokine secretion during *F. tularensis* challenge are key players in controlling the outcome of infection (Bosio et al., 2007; Mares et al., 2008; Sharma et al., 2009). Consequently, having shown that immunization of mice with mAb-*iFt* favors a pro-inflammatory cytokine profile secreted by PECs *in vitro*, the attempt was to determine the effect of our Fc γ R targeting approach on the levels of inflammatory cytokines in the lungs of immunized mice during the early stages of *F. tularensis* LVS infection. To accomplish this, C57BL/6 mice were immunized with PBS, or *iFt*, or mAb-*iFt*, boosted on day 21 and infected with a lethal dose of *F. tularensis* LVS on day 35 post-immunization. The lungs of euthanized mice were harvested after 24, 48 and 96 hours post-challenge, homogenized, and the IL-6, IL-10, TNF- α and IFN- γ cytokine levels were measured by direct sandwich ELISAs. The obtained results indicate a faster pro-inflammatory response in the lungs of mAb-*iFt* immunized mice compared to mice immunized with *iFt* alone, as assessed by the IL-6 and TNF- α production kinetics. Levels of IL-6 and TNF- α peaked at 48 hours post-infection, while they were significantly decreased by day 4 post-challenge (Fig. 5). This observation was accompanied by a drop of the bacterial load (data not shown). On the other hand, the pro-inflammatory cytokine levels tested continued to increase at 96 hours post infection in both the PBS and *iFt* immunized mice accompanied by an increase in the bacterial burden in the lungs (Fig. 5 and data not shown). Similar results among the three groups of mice were noted with IFN- γ (Fig. 5). On the contrary, the levels of IL-10

were significantly higher in mice immunized with either *iFt* or PBS versus mAb-*iFt* within the first 24 of infection, indicating the early anti-inflammatory properties of *F. tularensis* LVS. Importantly, the decrease of IL-10 in the mAb-*iFt* immunized mice observed at 96 hours post-infection was consistent with the previous observation, indicating that Fc γ R targeting shifts towards a pro-inflammatory cytokine profile at the early stages of *F. tularensis* infection (Fig. 2 and Fig. 5).

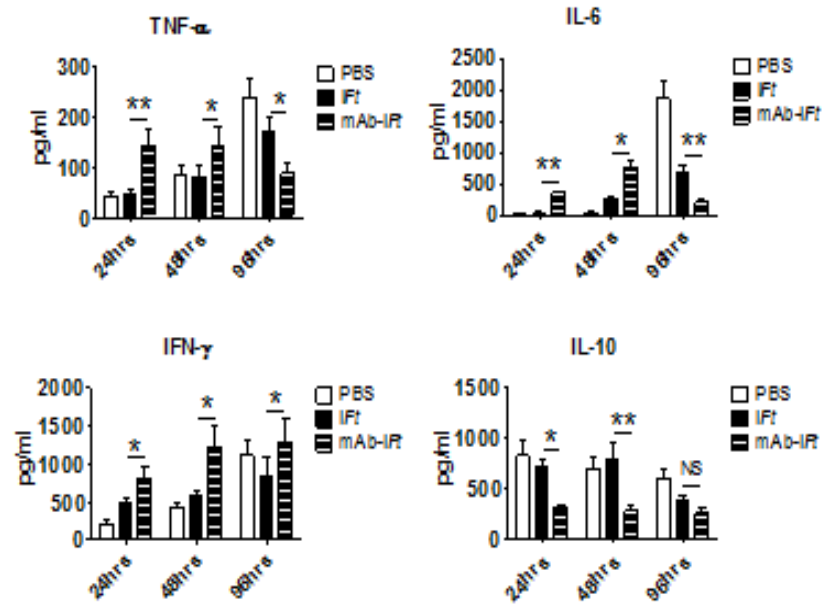


Fig. 5: Administration of mAb-iFt immune complexes reverses the anti-inflammatory properties of LVS in the lungs of immunized mice

C57BL/6 mice (n=6 for each group) were immunized i.n. with PBS, iFt (2×10^7 CFUs), or mAb-iFt, boosted on day 21 and challenged on day 35 with 10,000 CFUs of *F.tularensis* LVS. Lung tissue homogenates were obtained from immunized mice 24, 48 and 96 hours post-infection as indicated above and spun down at 15,000g for 30 minutes at room temperature to remove tissue debris. Cytokine levels were detected by using the IL-6, IL-10, TNF- α and IFN- γ ELISA kits and following vendor instructions (Biolegend). Results shown are mean \pm SD from three independent experiments determined by one-way ANOVA (* P-value < 0.1; ** P-value < 0.05).

FcγR targeting drives polarization of mouse macrophages towards the AM1 phenotype at the early stages of *F. tularensis* LVS infection

The results of these experiments have suggested so far that one of the immune evasion mechanisms exploited by *F. tularensis* is the reduction of pro-inflammatory cytokines during the early stages of infection. To further investigate possible mechanisms responsible for this shift in the innate immune response, the effect of FcγR targeting on macrophage activation and phenotype was analyzed in the lungs of immunized mice. Previous research showed that one of the ways of *F. tularensis* survival and replication within the host cell is its ability to alter the macrophage activation from classically activated alveolar macrophages (AM1) to alternatively activated alveolar macrophages (AM2) (Shirey et al., 2008). The AM1 macrophages are characterized by high levels of pro-inflammatory cytokines and thus, play an essential role in anti-bacterial innate immune response. In contrast, AM2 macrophages are associated with high levels of anti-inflammatory cytokines, in particular IL-10 (Katakura et al., 2004; Tomioka et al., 2012). Therefore, the flow cytometric analysis was performed to assess the number of AM1 and AM2 present upon immunization with mAb-iFt. C57BL/6 mice were immunized with PBS, iFt, or mAb-iFt, boosted on day 21 and infected with a lethal dose of *F. tularensis* LVS on day 35 post-immunization. The lungs of euthanized mice were harvested 24, 48 and 96 hours post-challenge and the levels of F4/80, CD11b, CCR7, MHC class II and B7.2 marker expression on white blood cells was analyzed by flow cytometry. Classically activated AM1 cells are characterized as F4/80⁺/CD11b^{int}/CCR7⁺/MHC classII⁺/B7.2⁺ cells, while AM2 cells were identified as

F4/80⁺/CD11b^{int}/CCR7⁻/MHC classII⁺/B7.2⁻ (Mantovani et al., 2004; Tomioka et al., 2012). The frequency and number of AM1 cells was significantly higher in mAb-i*Ft* immunized group of mice compared to mice immunized with i*Ft* alone, especially at the early stages of *F. tularensis* LVS infection 24 and 48 hours post challenge (Fig. 6). Interestingly, the levels of AM1 cells were comparable among the different immunized groups at 96 hours of post challenge. In addition, the data showed a significantly lower number of AM2 cells in the lungs of mAb-i*Ft* immunized mice relative to mice immunized with i*Ft* alone at all three time points post-infection. It is also of interest that mice immunized with PBS had the highest frequency of AM2 cells, proposing that AM2 polarization *in vivo* may be an additional immune evasion strategy of *F. tularensis*, especially at the early stages of infection.

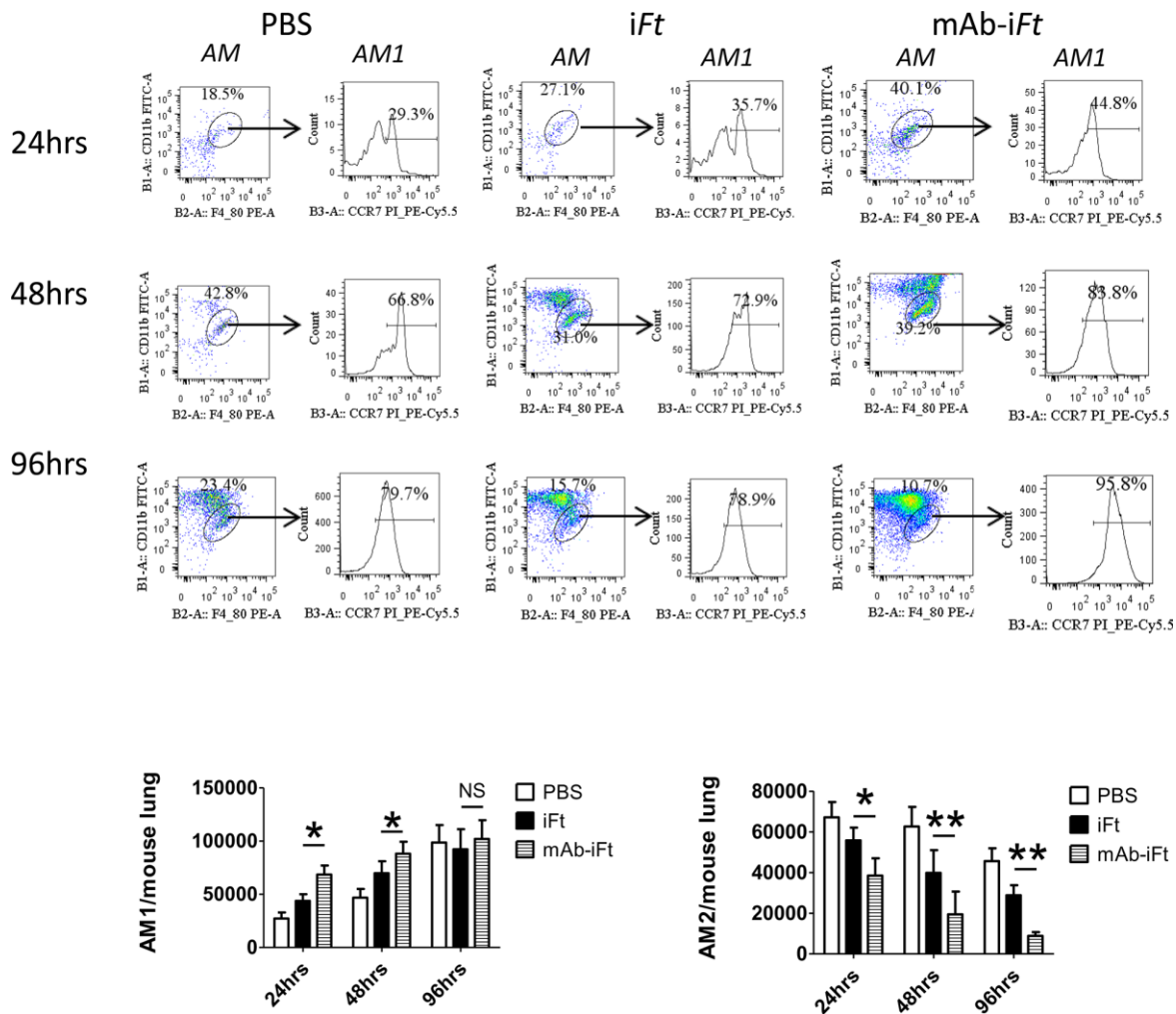


Fig. 6 Fc γ R targeting drives polarization of mouse macrophages towards the AM1 phenotype at the early stages of LVS infection

Lungs of immunized mice (n=6 for each group) were harvested 24, 48 and 96 hours post-infection. For cell surface marker staining, cells were washed with PBS-BSA-azide, resuspended in blocking buffer [PBS-BSA-azide plus 30 μ g/ml of normal mouse IgG (Sigma)] and incubated on ice for 30 minutes. Cells were then washed with PBS-BSA-azide and fluorescently labeled antibodies to CD11b, F4/80, MHC class II, B7.1, B7.2, CCR7, or their corresponding isotype controls were added. The cells were then incubated on ice for 30 minutes, washed, and then fixed with 2% paraformaldehyde. Cells were then analyzed by flow cytometry on an LSRII flow cytometer (BD Biosciences). Results shown are mean \pm SD from three independent experiments determined by one-way ANOVA (* P-value < 0.1; ** P-value < 0.05).

Enhanced maturation and activation of DCs in the lungs of mice immunized intranasally with mAb-*iFt* ICs

It has been previously demonstrated that targeting *iFt* to Fc γ Rs via the intranasal administration of pre-formed mAb-*iFt* ICs elicits protective responses against lethal challenges with *F. tularensis* LVS, and the category A *F. tularensis* agent, SchuS4 (Rawool et al., 2008; Czerkinsky et al., 2012). The protection observed was based on survival studies, bacterial burdens, and immunohistochemical analysis of mouse tissues. The protective effect was due to targeting of bacterial antigens to Fc γ R through opsonization with an anti-LPS IgG2a mAb. The antibody alone had no protective effect on mice against *F. tularensis* challenge. Furthermore, it was shown that targeting *iFt* to Fc γ Rs on APCs mediates internalization, processing, and presentation of the immunogen to *F. tularensis*-specific T-cell hybridoma *in vitro* (Rawool et al., 2008, Iglesias et al., 2012). Since DCs play a key role in processing and presenting antigens to T lymphocytes, effectively bridging the innate and adaptive immune system, experiments were designed to focus on the effect immunization with mAb-*iFt* ICs has on the number of activated DCs in the lungs of immunized mice. For this purpose, C57BL/6 mice were immunized with PBS, *iFt*, F(ab')₂-*iFt* or mAb-*iFt* and boosted on day 21. On day 35 post-immunization, lungs were harvested, digested, and the DCs were purified using lympholyte, followed by a one hour incubation at 37°C on petri dishes to negatively select for the adherent cells, such as macrophages. Following selection, we used the expression of CD11c and DEC-205 markers to define lung DCs (Witmer-Pack et al., 1995; Kronin et al., 2000; Bonifaz et al., 2004). All the selected cells that were CD11c⁺

were also positive for the dectin marker (DEC-205), indicating the DC purity of more than 98% (data not shown). The activation status of lung DCs was based upon the expression of MHC class II, B7.1, and B7.2 and the analysis was performed by flow cytometry. Interestingly, no significant differences were noted among the three groups of immunized mice (data not shown). In contrast, when immunized mice were infected with a lethal dose of *F. tularensis* LVS on day 35 post-immunization, and their DC profile was analyzed at different time points post-infection, there was a significant difference in the frequency and activation status of lung DCs between the *iFt* and mAb-*iFt* groups. More specifically, on days 2 and 5 post-infection there was a significantly higher number of CD11c⁺/DEC-205⁺ cells in the lungs of mAb-*iFt* immunized mice compared to mice immunized with *iFt* alone or F(ab')₂-*iFt* (Fig. 7B and 7C), potentially indicating increased recruitment of DCs in the lungs during infection. This difference was not significant on day 7 (Fig. 7D). It is also interesting to note that the overall white blood cell (WBC) cellularity in the lungs of mAb-*iFt* immunized mice was significantly increased following *F. tularensis* LVS challenge (Fig. 7A).

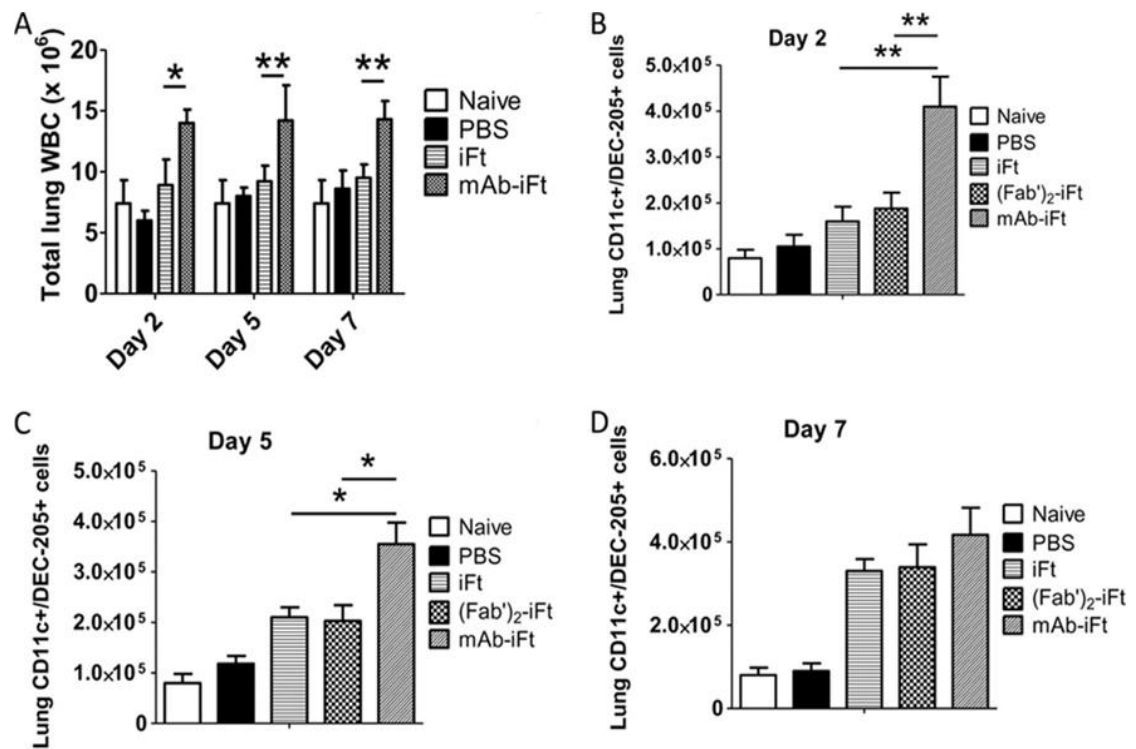


Fig. 7 Immunization of mice with mAb-iFt immune complexes enhances the number of CD11c⁺ cells in the lungs of immunized mice following *F. tularensis* LVS challenge

C57BL/6 mice (n=6 for each group) were immunized i.n. with PBS, iFt (1×10^9 CFUs), F(ab')₂-iFt or mAb-iFt, boosted on day 21 and challenged on day 35 with 20,000 CFUs of *F. tularensis* LVS. On days 2, 5, and 7 post-infection the lungs of immunized mice were harvested and the white blood cells were obtained and counted (A). Lungs were also harvested from unimmunized and uninfected mice (naïve) as a baseline control. The frequency of CD11c⁺/DEC-205⁺ cells was measured by flow cytometry and translated into absolute numbers for days 2 (B), 5 (C), and 7 (D) post-infection. Naïve mice were neither immunized nor challenged. Results shown are mean \pm SD from three independent experiments determined by one-way ANOVA and Student's t-test (* P-value < 0.1; ** P-value < 0.05). There was no significant difference between the two groups on day 7 post LVS infection.

On a similar note, the expression (Fig. 8) and absolute numbers (Fig. 9) of CD11c⁺ cells expressing MHC class II, B7.1, and B7.2 were significantly higher in the mAb-*iFt* immunized group compared to the group immunized with *iFt* alone or F(ab')₂-*iFt*, indicating for the first time that mucosal targeting of an immunogen to FcγR does not only enhance the frequency of DCs in the lungs upon subsequent infection, but also mediates *in vivo* DC maturation and activation enabling them to become better antigen presenting cells.

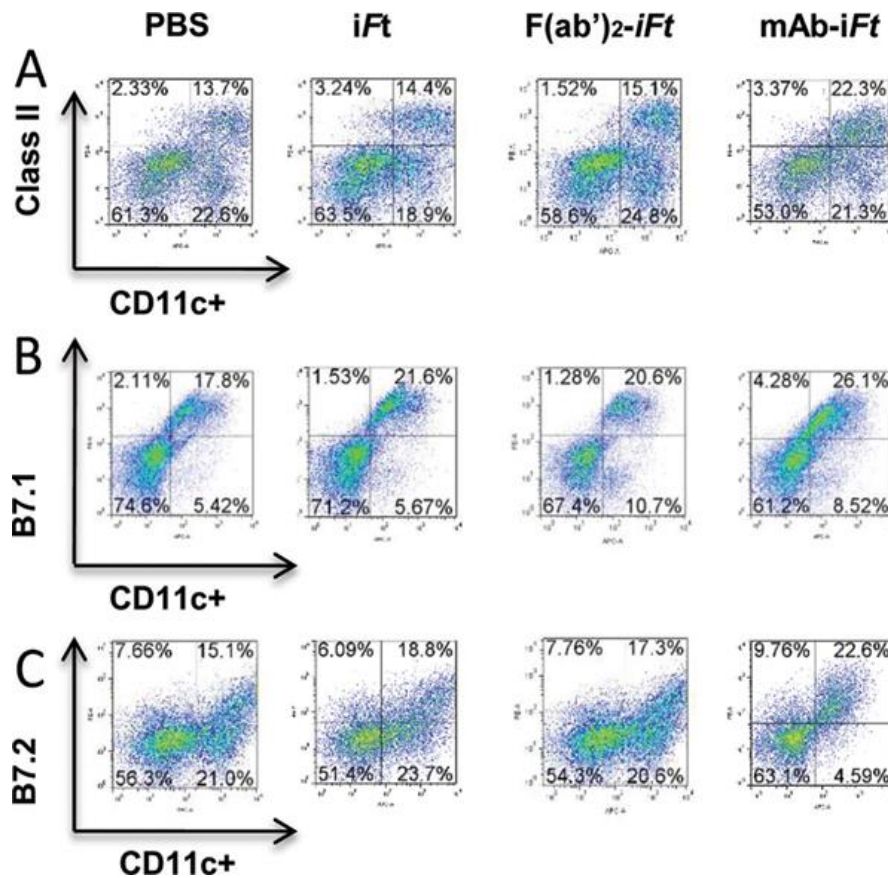


Fig. 8 Increased frequency of activated CD11c⁺ dendritic cells in the lungs of mice immunized with mAb-iFt immune complexes and challenged with *F. tularensis* LVS C57BL/6 mice (n=3 for each group) were immunized i.n. with PBS, iFt (1x10⁹ CFUs), F(ab')₂-iFt or mAb-iFt, boosted on day 21, and challenged on day 35 with 20,000 CFUs of iFt LVS. On day 2 post- LVS infection the lungs of immunized mice were harvested and the white blood cells were stained and analyzed by flow cytometry to obtain the expression levels of MHC class II (A), B7.1 (B), and B7.2 (C) on lung dendritic cells (CD11c⁺/DEC-205⁺). Results shown are representative of three independent experiments.

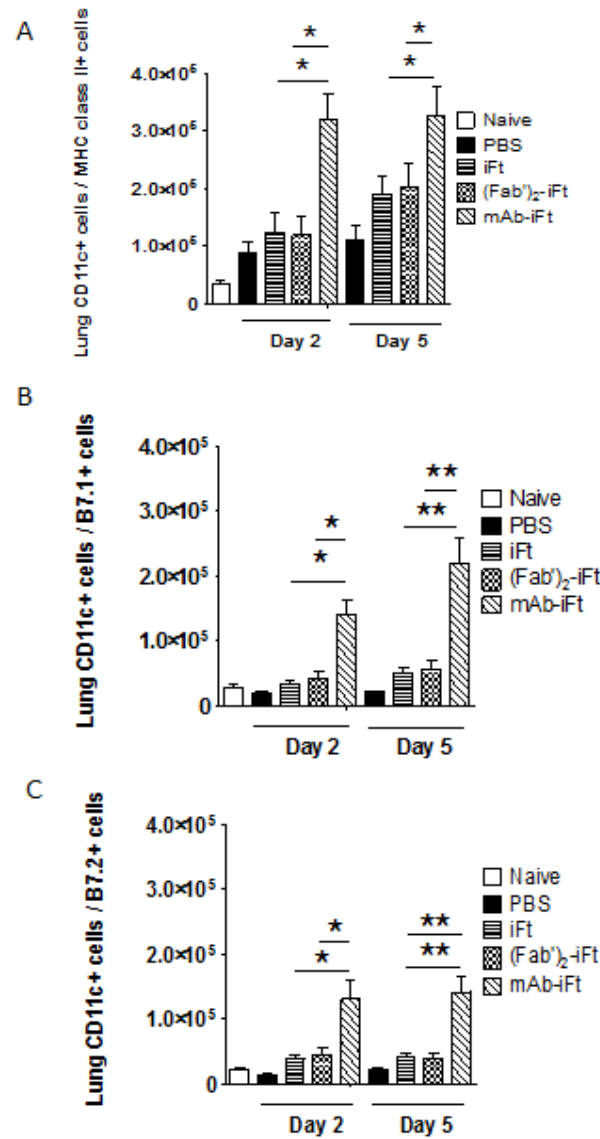


Fig. 9 Immunization of mice with mAb- iFt immune complexes increases the number of activated CD11c⁺ lung dendritic cells following *F. tularensis* LVS challenge

C57BL/6 mice (n=6 for each group) were immunized and challenged as in Fig. 8. On days 2 and 5 post- *F. tularensis* LVS infection the lungs of immunized mice were harvested and the white blood cells were obtained and counted. The frequencies of CD11c⁺/DEC-205⁺/MHC class II⁺ (A), CD11c⁺/DEC-205⁺/B7.1⁺ (B), and CD11c⁺/DEC-205⁺/B7.2⁺ (C) were translated into absolute cell numbers. Results shown are mean ± SD from three independent experiments determined by one-way ANOVA (* P-value < 0.1; ** P-value < 0.05).

Immunization with mAb-i*Ft* ICs enhances the generation of effector memory CD4⁺ T cells during *F. tularensis* LVS challenge

Memory T cell responses are often required to resolve intracellular bacterial infections, hence effective vaccines should have the ability to generate long-lasting, memory T cell populations. Memory cells are divided into central memory (CM) and effector memory (EM) cells based on their expression of cell surface receptors. More specifically, although both types of memory cells are positive for the adhesion molecule CD44, CM T cells are also positive for CD62L and the chemokine receptor CCR7, while EM T cells lack both (Sallusto et al., 1999; Kaech et al., 2002; Esser; et al., 2003).

It has been previously shown that targeting i*Ft* to FcγR via intranasal immunization with ICs generates protection against lethal *F. tularensis* challenge in an IFN-γ dependent fashion, indicative of a T cell recall response (Rawool et al., 2008). Having established that intranasal immunization with mAb-i*Ft* ICs increases the number of activated DCs in the lungs during *F. tularensis* LVS infection, and antigen presentation by DCs can effectively program T lymphocytes to differentiate into memory cells (Esser et al., 2003), an experiment was designed to determine the effect FcγR targeting on the generation of memory CD4⁺ T cells. For this purpose, C57BL/6 mice were immunized with PBS, or i*Ft*, F(ab')₂-i*Ft* or mAb-i*Ft* and boosted on day 21. On day 35 post-immunization, the lungs and spleens of immunized mice were harvested and the lymphocytes were stained with the relevant mAb followed by flow cytometric analysis as described in the Materials and Methods. In accordance with previous studies,

EM CD4⁺ T cells were characterized as CD3⁺/CD4⁺/CD44^{high}/CD62L^{low}/CCR7⁻, while the phenotype of CM memory CD4⁺ T cells was CD3⁺/CD4⁺/CD44^{high}/CD62L^{high}/CCR7⁺. Interestingly, regardless of the type of immunization, there was no generation of EM CD4⁺ T cells in the spleen or lungs of immunized mice (Fig. 10A). In fact, a similar population of CM T cells was present in all three groups, regardless of the type of immunogen used (Fig. 10A). Most importantly though, *F. tularensis* LVS infection of immunized mice generated a population of EM CD4⁺ T cells both in the lungs and spleens of mice within two days post infection (Fig. 10B). The levels of EM CD4⁺ T cells elicited during bacterial challenge were significantly higher in the mAb-i*Ft* immunized group compared to i*Ft* (Fig. 10B). The cells characterized as CM CD4⁺ T cells were also CCR7⁺ while the EM CD4 cells had very low levels of CCR7 expression (data not shown). The enhancement of the effector T cell population in the mAb-i*Ft* IC immunized group indicated that targeting antigens to FcγR can drive the generation of effector memory immune responses both locally (lung) and systemically (spleen) during bacterial infection. Mice immunized with F(ab')₂-i*Ft* ICs followed by *F. tularensis* LVS challenge, generated similar levels of EM CD4⁺ T cells compared to the i*Ft* immunized group, confirming that Fc receptor targeting of the immunogen is essential for this observation (Fig. 10B-10C). Importantly, the EM CD4⁺ T cells generated following Fc receptor targeting of i*Ft* were persistent for at least 25 days after *F. tularensis* LVS infection (Fig. 10B).

Interestingly, the population of central memory CD4⁺ T cells was negligible within two days following *F. tularensis* LVS infection in all three groups of mice (Fig.

10B). The qualitative (Fig.10B) and quantitative (Fig.10C) enhanced generation of effector versus central memory T cells observed in the mAb-*iFt* immunized group, suggests that EM CD4⁺ T cells have a more pivotal role in mediating protection against lethal *F. tularensis* LVS challenge over their CM counterparts.

Currently, the generation of effector vs central memory CD8⁺ T lymphocytes in *F. tularensis* model is being evaluated as this subpopulation of T cells have also been shown to play an important role in protection against this mucosal bacterial pathogen (Conlan et al., 1994; Yee et al., 1996).

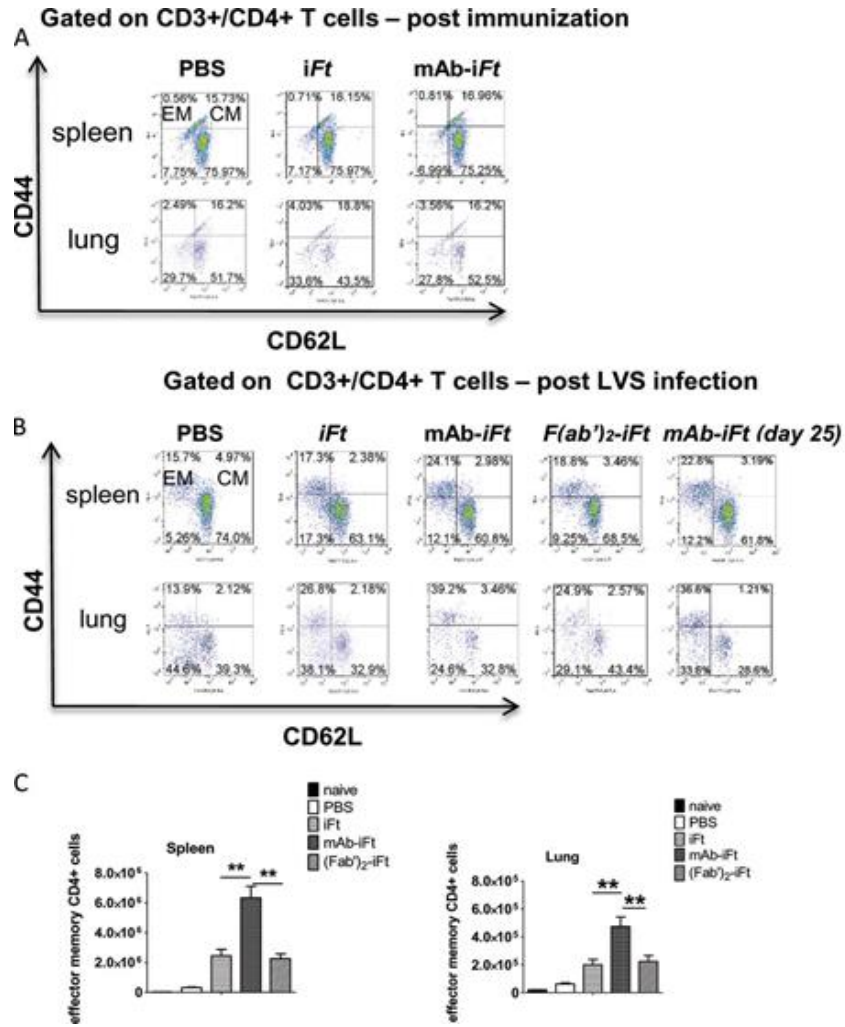


Fig. 10 Enhanced generation of effector memory CD4⁺ T cells in the spleen and lungs of mice immunized with mAb- iFt ICs, following *F. tularensis* LVS challenge
C57BL/6 mice (n=3 for each group) were immunized with PBS, iFt, mAb- iFt or F(ab')₂-iFt and challenged as described in Fig. 7. On day 35 post-immunization or on day 2 post-*F. tularensis* LVS challenge, the lungs and spleens of immunized mice were harvested and the white blood cells were obtained and counted. Expression levels of CD3⁺/CD4⁺/CD44⁺/CD62L⁺/CCR7⁺ T cells post-immunization (A) and post-infection (B), as well as their absolute numbers (C) were analyzed by flow cytometry. The effector memory cells (EM) are presented as CD44⁺ / CD62L⁻ while the central memory cells (CM) are presented as CD44⁺ / CD62L⁺. Results shown are mean ± SD from three independent experiments determined by one-way ANOVA and Student's t-test (* P-value < 0.1; ** P-value < 0.05).

IL-12 is necessary for protection against lethal *F. tularensis* LVS challenge following immunization with mAb-*iFt* ICs

The importance of T cells and IFN- γ in protection against *F. tularensis* LVS infection following immunization with mAb-*iFt* ICs provides evidence for the generation of a Th-1- like protective immune response. It is widely established that IL-12, secreted mainly by innate immune cells, such as DCs and macrophages, drives the differentiation of Th-1 effector T cells (Hsieh et al., 1993). Thus, it was hypothesized that the protective immune responses elicited following targeting of *iFt* to Fc γ R are dependent upon IL-12. In order to confirm this hypothesis, IL-12p35-deficient mice, and their control counterparts (C57BL/6), were immunized with PBS, *iFt*, or mAb-*iFt*, boosted on day 21, and infected with a lethal dose of *F. tularensis* LVS on day 35 post-immunization. The mice deficient for the p35 IL-12 subunit (as opposed to p40 deficient mice) have been selected due to the role of the p35 gene in the development of Th-1 responses (Liu et al., 2003). In addition, the p40 subunit is not unique to IL-12 as it is also shared by IL-23.

As anticipated, based on previous studies, 100% of the C57BL/6 mice that were immunized with mAb-*iFt* ICs survived the *F. tularensis* LVS challenge while immunization with *iFt* alone provided only 50% protection (Fig. 11A). IL-12p35-deficient mice though succumbed to infection within 13 days, regardless of the type of immunization indicating the importance of IL-12 in protection generated by Fc γ R targeting (Fig.11B). The role of IL-12 in protection against lethal *F. tularensis* challenge is evident in both the *iFt* and mAb-*iFt* immunized IL-12p35-deficient mice as both

groups completely succumbed to the infection. This indicates that targeting of the fixed bacterial immunogen to Fc γ R on APCs drives the production of higher levels of IL-12 during *F. tularensis* LVS challenge compared to *iFt* alone pointing towards quantitative versus qualitative difference between the two immunization approaches. In addition, there was significantly more lung DCs producing IL-12 from mAb-*iFt* IC immunized mice following *F. tularensis* LVS challenge, than from challenged mice immunized with *iFt* alone. The numbers of lung DCs positive for IL-12 from challenged mice immunized with the F(ab')₂-*iFt* ICs were also comparable to the *iFt* group, indicating that the increase in IL-12 production from lung DCs is Fc γ R-targeting dependent (Fig. 11C). Furthermore, culture of peritoneal cells (PECs) from mAb-*iFt* immunized and challenged mice with live *F. tularensis* LVS, led to significantly higher levels of IL-12p70 secretion as compared to PECs from challenged mice immunized with *iFt* alone (Fig. 11D). These data, collectively, present for the first time the importance of IL-12 in generating protective responses against *F. tularensis* LVS challenge when utilizing our Fc γ R targeting vaccine model.

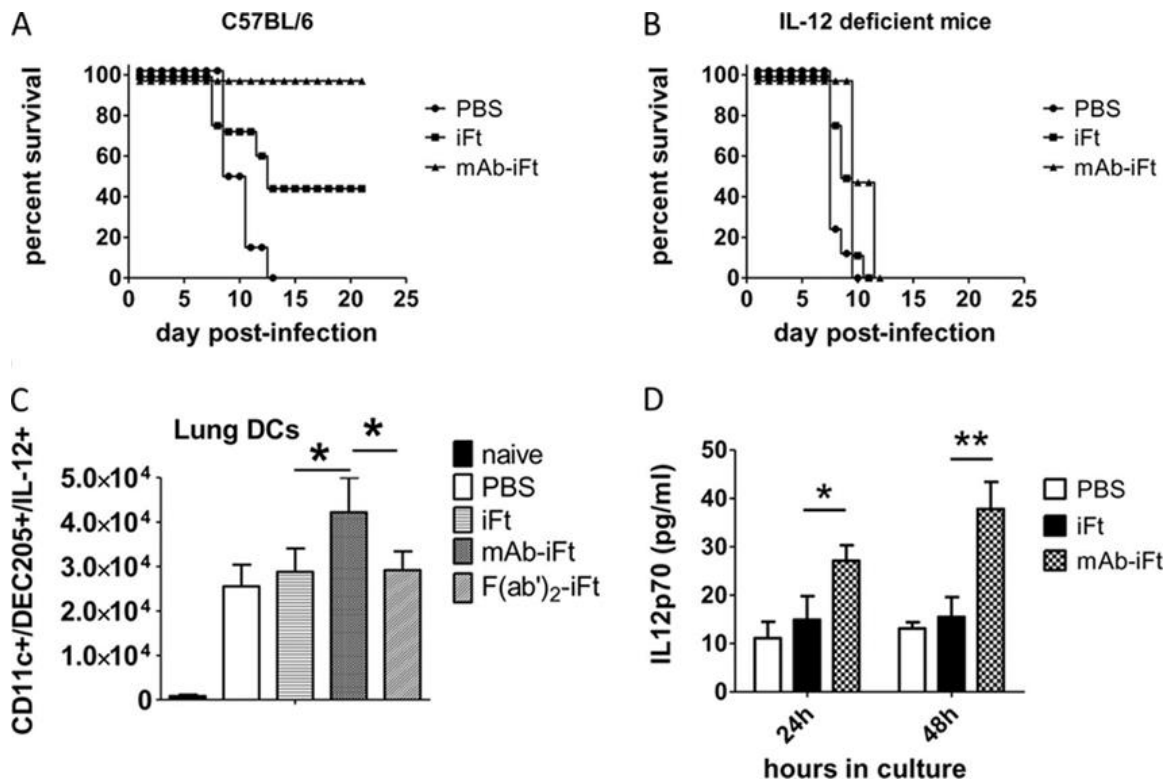


Fig. 11 Targeting of iFt to Fc γ R provides protection against *F. tularensis* LVS challenge in an IL-12-dependant fashion

C57BL/6 (A) or IL-12-deficient mice (B) ($n=6$ for each group) were immunized i.n. with PBS, iFt (1×10^9 CFUs), or mAb- iFt, boosted on day 21 and challenged on day 35 with 20,000 CFUs of *F. tularensis* LVS. Survival was monitored for 25 days post-infection and survival curves were generated using the Log-Rank (Mantel-Cox) test. The P- value for iFt versus mAb- iFt in A (C57BL/6) was 0.041. There was no significant difference between iFt and mAb- iFt immunized IL-12-deficient mice (B). C57BL/6 (C and D) mice were immunized i.n. with PBS, iFt (1×10^9 CFUs), mAb- iFt, or F(ab')₂- iFt, boosted on day 21 and challenged on day 35 with 20,000 CFUs of *F. tularensis* LVS. Two days post-infection lung DCs were obtained as previously described and stained intracellularly for IL-12 (C). Peritoneal exudate cells (PECs) were also obtained from all groups and cultured for 24 hours or 48 hours with *F. tularensis* LVS (1×10^3 CFUs /well). Supernatants were collected at designated time points and the levels of IL-12p70 were measured by CBA (D). Statistical analyses were carried out using the one-way ANOVA (* P-value < 0.1; ** P-value < 0.05).

IL-12 is required for the generation of effector memory CD4⁺ T cells upon intranasal immunization with mAb-*iFt* IC and subsequent *F. tularensis* LVS challenge

Having established that protection against *F. tularensis* LVS challenge via intranasal immunization with mAb-*iFt* ICs is IL-12 dependent, the role of the latter in the generation of effector memory CD4⁺ T cells following FcγR targeting was also examined. C57BL/6 mice were immunized i.n. with PBS, *iFt*, or mAb-*iFt*, boosted on day 21, and challenged on day 35 with a lethal dose of *F. tularensis* LVS. Mice were also injected i.p. with rat IgG2a anti-IL-12p35 or the isotype control on days 0, and 1 post-*F. tularensis* LVS infection. On day 2 after *F. tularensis* LVS challenge the levels of effector memory CD4⁺ T cells in the lungs and spleens of immunized mice were analyzed by flow cytometry. The data indicated that neutralization of IL-12 *in vivo* via the administration of anti-IL-12 mAb significantly reduced the frequency and number of effector memory CD4⁺ T cells both in the lungs and spleen of immunized mice (Fig. 12A and 13A, respectively). In accordance with these results, immunization of IL-12-deficient mice with *iFt* or mAb-*iFt* ICs, followed by lethal LVS infection, failed to enhance the generation of effector memory CD4⁺ T cells in both the lungs and the spleens of immunized mice (Fig. 12B and 13B). Altogether, these data suggest that the necessity of IL-12 for protection against lethal *F. tularensis* LVS challenge is, in part, due to the ability of IL-12 to generate EM CD4⁺ T cells, following targeting of bacterial immunogens to FcγR.

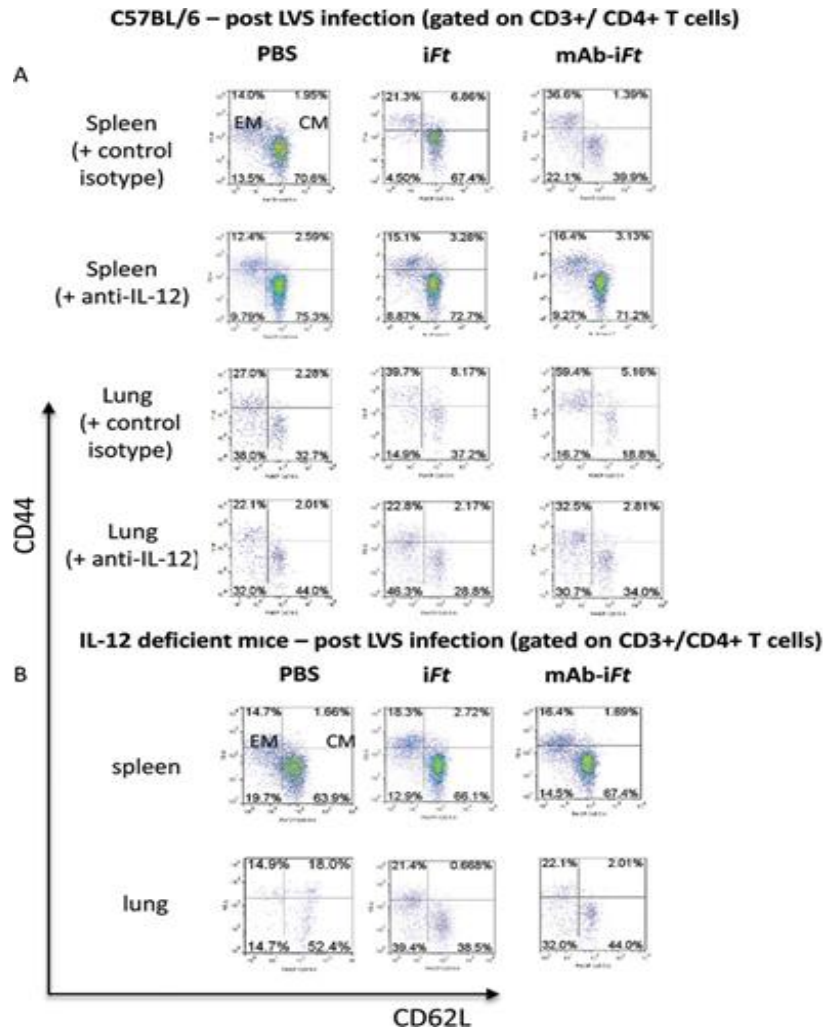


Fig. 12 Generation of effector memory CD4⁺ T cells following targeting of iFt to FcγR, during *F. tularensis* LVS challenge, is IL-12-dependant

C57BL/6 (A) or IL-12-deficient mice (B) (n=4 for each group) were immunized and challenged as described in Fig. 11. C57BL/6 mice were also injected i.p. with rat IgG2a anti-IL-12 (500mg/dose), or the control isotype, on days -1, 0, and 1 post-LVS infection. On day 2 after *F. tularensis* LVS challenge, the lungs and spleens of immunized C57BL/6 mice (A) and IL-12-deficient mice (B) were harvested and the white blood cells were obtained and counted. Expression levels of CD3⁺/CD4⁺/CD44⁺/CD62L⁺/CCR7⁺ T cells in the lungs and spleens of immunized C57BL/6 mice (A) and IL-12-deficient mice (B) were obtained by flow cytometry. The effector memory cells are presented as CD44⁺ / CD62L⁻ while the central memory cells are presented as CD44⁺ / CD62L⁺. Results are representative of three independent experiments.

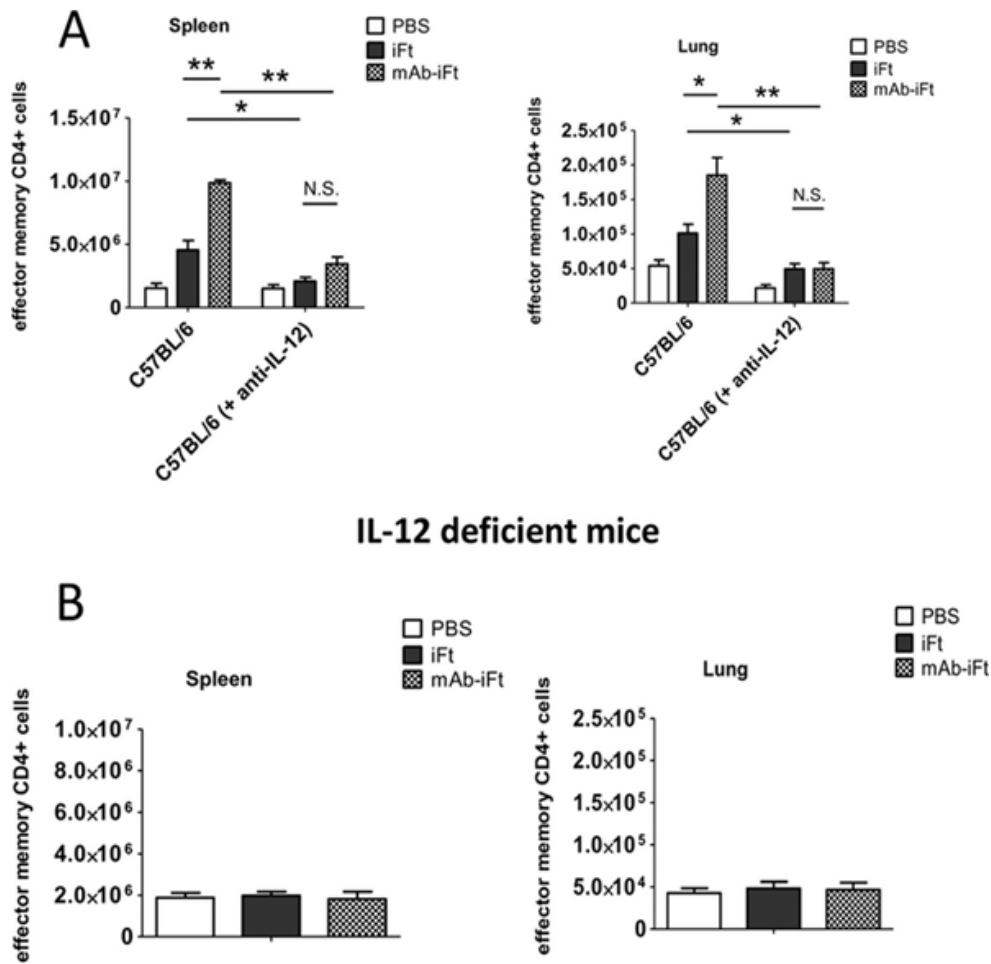


Fig. 13 Immunization of mice with mAb- iFt immune complexes increases the absolute numbers of effector memory CD4⁺ T cells, during *F.tularensis* LVS infection, in an IL-12-dependant fashion

Immunization and challenge studies were conducted in C57BL/6 (A) or IL-12-deficient mice (B) (n=4 for each group) as in Fig. 11. On day 2 after *F. tularensis* LVS challenge, the lungs and spleens of immunized mice were harvested and the lymphocytes were obtained and counted. C57BL/6 mice were also injected i.p. with rat IgG2a anti-IL-12 (500mg/dose), or the control isotype, on days -1, 0, and 1 after LVS infection. Expression levels of CD3⁺/CD4⁺/CD44⁺/CD62L⁺/CCR7⁺ T cells in the lungs and spleens of immunized C57BL/6 mice (A) and IL-12-deficient mice (B) were obtained by flow cytometry and their absolute numbers were calculated. Results shown are mean \pm SD from three independent experiments. Statistical analyses were carried out using the one-way ANOVA (* P-value < 0.1; ** P-value < 0.05).

Enhanced generation of IFN- γ secreting CD4⁺ effector memory T cells during *F. tularensis* LVS challenge in mice immunized i.n. with mAb-iFt ICs

The importance of IFN- γ in protection against *F. tularensis* LVS challenge, and the enhanced generation of effector CD4⁺ memory T cells following mAb-iFt immunization, led to hypothesis that the latter cell population is involved in the production of IFN- γ during *F. tularensis* LVS infection. To test this, C57BL/6 mice were immunized i.n. with PBS, iFt, or mAb-iFt, boosted on day 21, and challenged on day 35 with a lethal dose of *F. tularensis* LVS. Two days post-challenge, splenocytes from immunized mice were obtained and cultured for 5 hours in the presence or absence of *F. tularensis* LVS, while PMA and ionomycin were used as positive controls. Following incubation, cells were collected and pooled from each group, stained for the extracellular markers CD4/CD44/CD62L/CCR7 and intracellularly for IFN- γ . As depicted in Figure 14, there were significantly more *F. tularensis*-specific CD4⁺ splenocytes from mAb-iFt immunized mice that secreted IFN- γ upon *in vitro* re-stimulation. The CD4⁺ / IFN- γ ⁺ double positive cells also possessed the EM phenotype (CD44⁺/CD62L^{low}/CCR7⁻). These observations indicate that the source of IFN- γ , essential for protection against *F. tularensis* LVS challenge, is at least in part the effector memory CD4⁺ T cells generated following i.n. immunization with mAb-iFt ICs. Surprisingly, the CD4⁺/IFN- γ ⁺ population observed were not CD8⁺ T lymphocytes (data not shown).

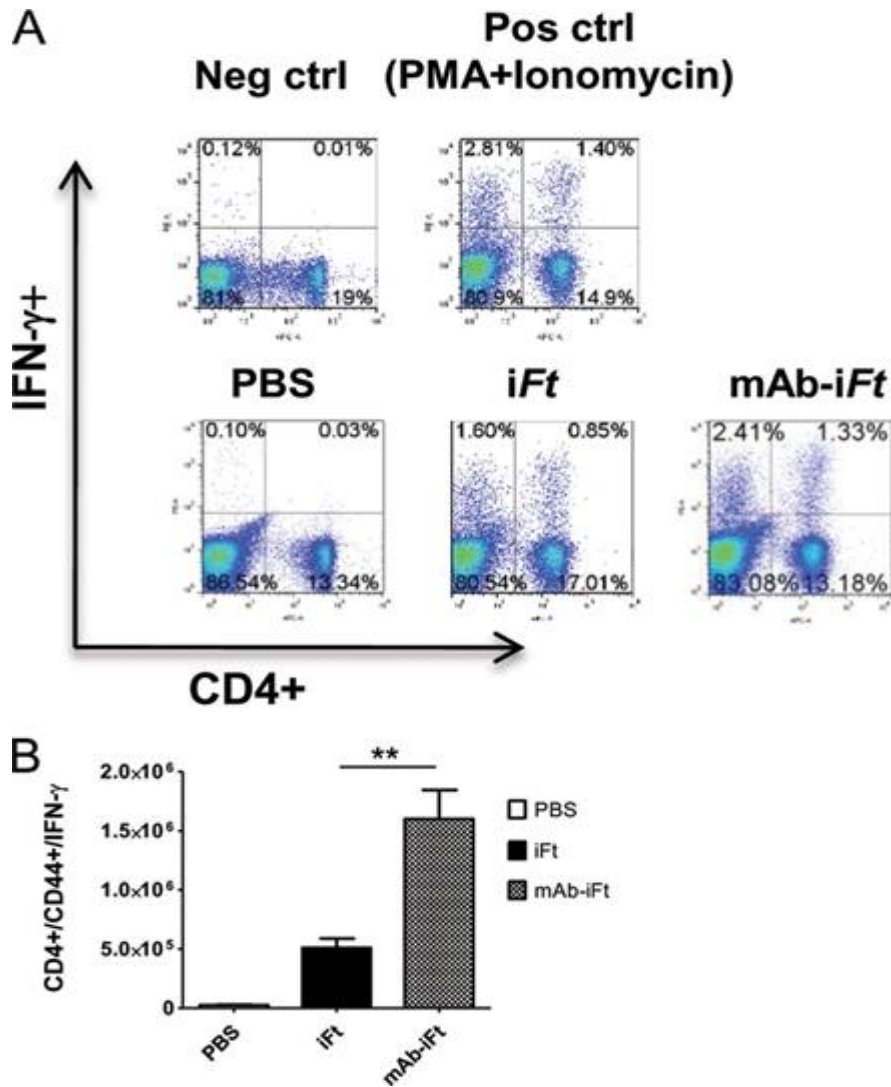


Fig. 14 Increased generation of IFN- γ producing effector CD4⁺ T cells in mice immunized with mAb- iFt IC compared to mice immunized with iFt alone

C57BL/6 (n=4 for each group) were immunized and challenged as described in Fig. 7. On day 2 after *F. tularensis* LVS challenge, the spleens were harvested and splenocytes were cultured for 5 hours with *F. tularensis* LVS (1×10^3 CFUs per 2×10^5 SPCs/well). Following incubation cells from each group were pooled, counted, and stained extracellularly for CD4, CD44, CD62L and CCR7 and intracellularly for IFN- γ . Analysis (A) and enumeration (B) of IFN- γ ⁺ effector CD4⁺ T cells were done by flow cytometry. Data shown are mean \pm SD from two independent experiments determined by Student's t-test. The P- value for the iFt versus the mAb- iFt group in panel B was 0.03 (* P-value < 0.1; ** P-value < 0.05).

Discussion

Francisella tularensis is a Gram-negative intracellular bacterium that causes tularemia, a disease that could potentially be fatal in humans and animals. It can be transmitted through insect bites, infected carcasses, contaminated water, and inhalation of contaminated air (Metzger et al., 2007). Because of its high infectivity the Centers for Disease Control and Prevention designates *F. tularensis* as a Category A biological agent (Rotz et al., 2002). The search for a safe and effective vaccine against infectious tularemia is still ongoing due to the lack of clear understanding of its pathogenesis. One of the major questions is to understand pathobiology of the bacterium and to evaluate the host's immune responses in the early stages of infection.

The current study demonstrated for the first time that targeting of inactivated *F. tularensis* (iFt) bacteria to Fcγ receptors via formation of immune complexes reverses the potential detrimental effect of IL-10 during the early stages of *F. tularensis* infection. Due to the lack of an identified *F. tularensis* protective antigen, the focus was on an inactivated vaccine, which has been shown to protect against highly virulent SchuS4 organism in the highly stringent animal model C57BL/6 (Rawool et al., 2008).

Early control of bacterial infection depends on the secretion of pro-inflammatory cytokines that regulate the activation of antigen presenting cells and the generation of a protective Th-1-like response (Medzhitov & Janeway, 1997; Pashine et al., 2005; Kalinski, 2012). One of the characteristics of *F. tularensis* infection is the lack of such responses during early progression of infection. While some studies depict that this

effect is due to the low endotoxicity of *F. tularensis* lipopolysaccharide, which is structurally different from other Gram-negative bacteria (Ancuta et al., 1996; Barker et al., 2006; Duenas et al., 2006; Hajjar et al., 2006), other studies show that the potential up-regulation of anti-inflammatory cytokines is an important factor in the progression of *F. tularensis* infection (Woolard et al., 2007; Metzger et al., 2013). The latter could possibly be due to suppression of the early activation of inflammasome by *F. tularensis*, one of the mechanisms that the bacteria utilizes to evade the early immune response (Huang et al., 2010; Ulland et al., 2010; Dotson et al., 2013).

The anti-inflammatory cytokine IL-10 plays a major role in suppression of host immune system by inhibiting the antigen presentation ability of macrophages and dendritic cells mainly by down-regulating the expression of CD80, CD86 and MHC class II molecules, as well as the production of pro-inflammatory cytokines (Pestka et al., 2004). The ability of IL-10 to suppress innate, inflammatory responses against intracellular pathogens has been previously reported. For instance, lack of endogenous IL-10 production led to a decrease in number of *Trypanosoma cruzi* parasites in the blood of infected mice (Abrahamsohn & Coffman, 1996). Similarly, lack of IL-10 resulted in higher resistance of mice to *Listeria monocytogenes* (Dai et al., 1997). In addition, viruses have also evolved mechanisms to escape the immune response by altering the Th1/Th2 balance. Thus, it has been shown that elevated levels of IL-10 correlated with higher viral load in HIV infected individuals (Brockman et al., 2009).

IL-10 has also been identified as a key regulator of immune response to *F. tularensis* infection (Metzger et al., 2013). In particular, IL-10 mediates suppression of

IL-17, a cytokine that is essential in the initiation of a protective immune response to *F. tularensis* infection (Leiby et al., 1992; Elkins et al., 2003; Duckett et al., 2005; Lin et al., 2009).

It is well established that targeting of antigen to FcγRs using a receptor subclass-specific monoclonal antibodies increases the binding, internalization and processing of an antigen by antigen presenting cells (APCs) in the absence of an adjuvant (Gosselin et al., 1992). Furthermore, targeting of inactivated *F. tularensis* bacteria to FcγRs through intranasal immunization of mice with mAb-*iFt* enhances immune response and protection against *F. tularensis* infection possibly via FcγR dependent enhanced uptake of the antigen by APCs and further presentation to T cells leading to enhanced T cell activation (Rawool et al., 2008; Iglesias et al., 2012). The current study have showed that a potential mechanism of protection in the *F. tularensis* model utilized, following FcγR targeting of fixed bacteria, entails the generation of a Th-1 response during the early phases of *F. tularensis* infection, by inhibiting the synthesis of the anti-inflammatory cytokine IL-10.

Numerous studies have demonstrated that during *F. tularensis* infection the early pro-inflammatory responses are significantly suppressed Bosio et al., 2007; Walters et al., 2013), followed by overwhelming up-regulation after 48 hours of infection leading to a severe sepsis (Mares et al., 2008; Sharma et al., 2009). The results of these current experiments show that *iFt* targeting to FcγRs up-regulates pro-inflammatory responses at early stages of *F. tularensis* infection both *ex vivo* and *in vivo*. Particularly, immunization of C57BL/6 mice with mAb-*iFt* shifted the cytokine profile towards a pro-inflammatory

Th-1 type in the lungs and peritoneum of immunized mice during LVS infection, which was associated with reduction in bacterial load. Conversely, immunization with PBS and the inactivated immunogen alone resulted in considerably lower amounts of Th1 cytokines and higher amounts of IL-10 synthesis accompanied by an increase in bacterial burden in the lungs of infected mice. These observations indicate that immunization of animals with mAb-iFt elicits a robust, pro-inflammatory immune response in the early phases of *F. tularensis* infection by reversing the inhibitory effect of IL-10 both locally and systemically.

The components of *F. tularensis* that contribute to the inhibition of pro-inflammatory responses are not completely understood yet. However, it is well established that *F. tularensis* LPS shows low endotoxic activity due to its structural differences from other intracellular pathogens (Ancuta et al., 1996; Barker et al., 2006; Duenas et al., 2006; Hajjar et al., 2006). These findings that *F. tularensis* LPS elicits inefficient pro-inflammatory response and up-regulates IL-10 synthesis indicate that LPS is partly responsible for the anti-inflammatory activities of *F. tularensis* LVS. The importance of pro-inflammatory cytokines, in particular IFN- γ , in controlling the intracellular infection has been described in previous reports. IFN- γ deficiency in mice during *Listeria monocytogenes* infection compromised activation of macrophages allowing bacteria to escape from phagolysome and further replicate within cytoplasm (Dai et al., 1997). Similarly, lack of IFN- γ resulted in decreased survival of mice and increase in bacterial burden during *Legionella pneumophila* infection (Shinozawa et al., 2002). In addition, IFN- γ dependent activation of human monocyte-derived macrophages

inhibited escape of *F. tularensis* into the cytoplasm, thus preventing bacterial replication (Santic et al., 2005). In the current study it was observed that the increased IL-10 synthesis in the early stages of *F. tularensis* infection coincided with a decrease of the pro-inflammatory cytokines IL-12p70 and TNF- α in the absence of endogenous IFN- γ . Addition of exogenous IFN- γ to cultures of LPS-treated PECs, however, was able to reverse this suppression. Likewise, an increase in the synthesis of IL-12p70 and TNF- α was observed in IL-10 deficient mice after stimulation with *F. tularensis* LPS. Therefore, these data demonstrate that early up-regulation of IL-10 is one of the means of Th-1 immune response suppression during early stages of *F. tularensis* infection, enabling the bacteria to avoid classical anti-bacterial mechanisms.

The source of pro-inflammatory cytokines during infection is primarily the professional antigen presenting cells, such as dendritic cells and macrophages. Proper activation and differentiation of macrophages and dendritic cells is important for the generation of robust, innate immune response against bacterial pathogens. Depending on the extracellular cytokine background, the activated macrophages are divided into two distinct groups, classically activated macrophages (AM1) associated with high levels of pro-inflammatory cytokines and alternatively activated macrophages (AM2) characterized by increased levels of anti-inflammatory cytokines, in particular IL-10 (Benoit et al., 2008). In fact, IL-10 and CCL17, both produced by AM2, are key players in suppressing induction of AM1 cells (Katakura et al., 2004). Shirey et al. (2008) demonstrated that *F. tularensis* LVS skews macrophage activation from anti-bacterial AM1 phenotype towards AM2, which allows its survival and uncontrolled replication

within host cells (Shirey et al., 2008). Based on these findings, the hypothesis was that targeting of mAb-*iFt* may alter the macrophage phenotype from anti-inflammatory AM2 to pro-inflammatory AM1. The data showed that immunization of mice with mAb-*iFt* resulted in higher frequency and number of AM1 cells compared to mice immunized with *iFt* alone. In addition, a significantly lower number of AM2 cells in the lungs of mAb-*iFt* immunized mice were observed relative to mice immunized with *iFt* alone. Interestingly, immunization with PBS resulted in the highest frequency of AM2 cells, suggesting that AM2 polarization *in vivo* may be an additional immune evasion strategy of *F. tularensis*, especially at the early stages of infection.

Further, it was demonstrated that this vaccination strategy increases the frequency and activation of DCs in the lungs of immunized mice during *F. tularensis* infection, while it enhances the generation of effector memory CD4⁺ T cells, both locally (lung) and systemically (spleen), in an IL-12-dependent fashion. This effector memory cell population is believed to play an important role in the protection observed against *F. tularensis* LVS challenge, partly via its increased ability to produce IFN- γ . Although the antibodies tend to form bacterial aggregates, the findings of this study depend upon specifically targeting antigens to Fc γ Rs. In particular, it was observed that the immunization with F(ab'₂) mAb-*iFt* ICs failed to enhance recruitment and activation of DCs in the lungs of immunized mice as well as to generate effector memory CD4⁺ T cells during infection. These results are in agreement with previous reports where subdued immune responses were observed when utilizing immune complexes lacking Fc domain (Rawool et al., 2008; Iglesias et al., 2012).

Modulating DCs, one of the most important professional APCs of the immune system, presents an attractive target for vaccine design. Indeed, DCs migrating from the periphery to secondary and tertiary lymphoid organs, loaded with exogenous antigens and activated by “danger signals”, identify naïve T cells and induce their proliferation and differentiation to effector and memory phenotypes. Indeed, the superior capacity of DCs to activate naïve T cells following nasal and oral immunizations has been determined in several studies (Vermaelen et al., 2001; Anjuere et al., 2004; Chirido et al., 2005; Wikstrom et al., 2006; Jaensson et al., 2008). In the current study, targeting *iFt* to FcγR via intranasal immunization of mice with mAb-*iFt* ICs increased the overall white blood cell cellularity of immunized mice during *F. tularensis* LVS infection compared to mice immunized with *iFt* alone. Most importantly, the frequency and activation status of lung DCs was also significantly increased post-challenge. Mice immunized with F(ab')₂ – *iFt* ICs failed to recruit and increase the activation of DCs in the lungs, indicating that this effect is dependent upon targeting of the immunogen to FcγRs. However, it is currently unclear whether expression of FcγR is required for DCs recruitment and activation. Nevertheless, the enhanced generation of activated DCs in the lungs of mice immunized with ICs provides evidence of enhanced mucosal immunity.

Although it has been previously established that FcγR targeting of a fixed bacterial immunogen (*iFt*) *in vitro* leads to DC activation (Iglesias et al., 2012), the exact mechanism that leads to their *in vivo* maturation and potential recruitment in the lungs remains unclear. At different developmental stages and at various anatomical sites DCs may express various chemokine receptors including: CCR1, CCR2, CCR4, CCR5,

CCR6, CCR7, CXCR1, and CXCR4 (Dieu et al., 1998; Cyster, 1999; Sallusto et al., 1999(a); Sallusto et al., 1999(b); Sallusto et al., 1999(c)). These findings suggest that chemokines and chemokine receptor expression levels may regulate DC trafficking. Indeed, in various infection models, including *Mycobacterium bovis*, *Schistosoma mansoni*, and *Cryptococcus neoformans*, expression of CCR2 in mice was necessary for DC recruitment and activation (Chiu et al., 2004; Osterholzer et al., 2008). Similarly, in two *F. tularensis* studies using *F. tularensis* LVS, CCR7⁺ DCs were found to be recruited in areas of high CCL19 and CCL21 chemokine concentrations (Bar-Haim et al., 2008; Chiavolini et al., 2010). Whether the levels of these chemokines are increased significantly in the lungs of IC immunized mice over mice administered with *iFt* alone, essentially recruiting mature DCs from peripheral sites remains to be addressed.

Chemokine expression also plays a role in the recruitment of CD4⁺ T cells at the sites of infection where they are exposed to concentrated amounts of polarizing cytokines, such as IL-12, secreted by the activated DCs (Osterholzer et al., 2008). In the current study, lung DCs from mAb-*iFt* IC immunized mice had an increased ability to secrete IL-12 upon re-stimulation *in vitro* compared to mice immunized with the untargeted *iFt*. The activated DCs would efficiently present *F. tularensis* antigens to CD4⁺ T cells polarizing them towards a Th-1 phenotype via secretion of IL-12 and driving their differentiation towards a protective effector memory phenotype. Furthermore, the observation that PECs showed increased secretion of IL-12 upon re-stimulation *in vitro* is of great interest as it depicts the ability of this vaccine strategy to elicit both mucosal and systemic protective immune responses. Indeed, immunization of

mice with mAb-*iFt* ICs increased the expression of MHC class II and co-stimulatory molecules (B7.1 and B7.2) on the surface of lung DCs *in vivo*, hence facilitating a more robust antigen presentation to T lymphocytes and subsequent generation of memory. It is believed the activation of lung DCs is a direct effect of FcγR targeting and is not aided by the LPS present in the bacterial cell wall. This is evident due to the significant differences in DCs maturation between the *iFt* and mAb-*iFt* immunized mice. In addition, *F. tularensis* LPS is structurally different from other enteric LPS and has reduced stimulatory effect on APCs (Ancuta et al. 1996; Duenas et al., 2006).

Although it is clear that memory T cells can be distinguished into central and effector subtypes, their relevance during recall responses has been controversial. Central memory (CM) T cells usually reside in the secondary lymphoid tissues due to their high expression of CD62L and CCR7. Effector memory (EM) T cells on the other hand, have a greater capacity to migrate to inflamed tissues, partly due to increased expression of chemokine receptors, such as CCR2 and CCR5 (Gunn et al., 1998; Sallusto et al., 1999 (a); Campbell & Butcher, 2000; Kaech et al., 2002; Esser et al., 2003; McKinstry et al., 2010). Generation of memory lymphocytes is often used to assess vaccination strategies, especially against intracellular pathogens. It has been previously shown that immunization with mAb-*iFt* immune complexes provides protection against *F. tularensis* infection 30 days post-immunization, indicating that targeting of bacterial antigens to FcγR results in the generation of protective memory lymphocytes (Rawool et al., 2008). The lack of an increased CM T cell population though following FcγR targeting, was surprising. Perhaps the significant enhancement of EM T cells during *F. tularensis* LVS

infection is of vital importance for the protection observed in animal model used in this study. Indeed, it has been shown that at early stages after bacterial infection, effector cells dominate the memory pool and provide potent protective immunity (Roberts et al., 2005).

The origin of the EM CD4⁺ T cells in the current study remains unclear. One possibility is that these cells were generated in the secondary lymphoid organs, such as spleen and lymph nodes, and homed to the lungs early, after *F. tularensis* LVS challenge, possibly in a chemokine-dependent manner. Alternatively, the EM T cells may have been generated locally, from the central memory pool. Indeed, in an elegant study from Blander et al., (2003), use of a transgenic T cell line showed that a significant percentage of CM T lymphocytes can acquire effector functions upon re-stimulation (Blander et al., 2003). Although the levels of CM T cells in the lungs and spleens of IC immunized mice were not different from the levels observed in mice immunized with *iFt* alone, it is speculated that the differences at the post-immunization stage are qualitative. It is likely that the enhanced activation status of DCs enable them to generate CM T cells that are more effectively programmed to switch to an effector memory phenotype during infection, both in the mucosa and systemically. Therefore, it is clear that the ability of vaccines to induce EM T cells is a requirement for protection. In addition, generation of EM cells in the secondary lymphoid organs requires prolonged and systemic exposure of the immunogen which often poses a challenge, especially for non-replicative vaccine vectors. It is possible that targeting *iFt* to FcγR aids the widespread distribution of

antigen, increasing the number of antigen encounters by naïve T cells and thus circumventing this problem.

Currently it is not certain whether the EM CD4⁺ T cells are independently capable to provide protection against pulmonary infection with *F. tularensis*. Previous studies have implicated both CD4⁺ and CD8⁺ T cell subtypes in protection against this mucosal pathogen as neutralization of either CD4⁺ or CD8⁺ T cells *in vivo* was not detrimental to the host (Conlan et al., 1994; Yee et al., 1996). One can assume that protection mediated by CD4⁺ T cells is mainly IFN- γ dependent while the cytotoxic effects of CD8⁺ T lymphocytes will be important in clearing the infection. The independent protective role of CD4⁺ T cells in the model used is currently under investigation via adoptive transfer experiments, while the effect of mAb-*iFt* IC immunization on the generation of CD8⁺ memory T lymphocytes is also currently being studied.

Another aspect to be considered in vaccine design is the maintenance of effector memory cells. Apart from persistence of the immunogen, the cytokine milieu may mediate the long-term survival of these memory cells. More specifically, Esser *et al.*, (2003), suggested that IL-12 may be a candidate factor to maintain CD4⁺ memory cells (Esser et al., 2003). In addition, IL-12 has been shown to enhance the potency and increase the durability of a leishmanial protein vaccine in mice (Gurunathan et al., 1998). In this study, IL-12 was necessary in protection against lethal *F. tularensis* LVS challenge as well as for generating EM CD4⁺ T cells. This is in accordance with previous studies showing that both subunits of IL-12 (p35 and p40) are essential in resolving pulmonary *F. tularensis* infection while the administration of recombinant IL-

12 i.n. facilitates bacterial clearance from the lungs of infected mice (Elkins et al., 2002; Pammit et al., 2004; Duckett et al., 2005). Therefore, it is likely that targeting *iFt* to Fc γ R on DCs (and potentially macrophages as well) increases the levels of IL-12 *in vivo* which drives EM T cell differentiation that is long-lived and displays Th-1-phenotype characteristics. In the current model, EM CD4⁺ T cells from IC immunized mice had higher frequency as well as increased ability to secrete IFN- γ compared to cells obtained from mice immunized with *iFt* alone, upon *in vitro* re-stimulation with *F. tularensis* LVS. It is believed that the CD4⁺ EM T-lymphocytes generated in this vaccine platform are also long lived as significant populations of these cells were still detectable 25 days post *F. tularensis* LVS challenge. The presence of IFN- γ in the memory CD4⁺ T lymphocytes following *in vitro* re-stimulation with *F. tularensis* LVS indicates specificity against *F. tularensis*. Enhanced production of IFN- γ *in vivo* can activate macrophages thus facilitating bacterial clearance while driving the switch towards Th-1 like antibody isotypes, such as IgG2a or IgG2c. Indeed, a Th-1 like antibody response against *F. tularensis* has been previously shown following targeting of bacterial antigens to Fc γ R (Rawool et al., 2008). Hence, these observations indicate that Fc γ R targeting mediates Th-1 polarization of EM T lymphocytes through IL-12 secretion, while it generates a greater frequency of antigen-specific memory T cells. The lack of protection against lethal *F. tularensis* LVS challenge following immunization of IFN- γ deficient mice with mAb-*iFt* IC (Rawool et al., 2008), leads to conclusion that the ability of EM T cells to produce IFN- γ may be indispensable for protection in this immunization model.

The specific *F. tularensis* responses observed in this study also revealed a CD4⁺ cell population that expressed IFN- γ during re-stimulation with *F. tularensis* LVS *in vitro*. This population had higher frequency in the mAb-i*Ft* immunized mice, suggesting a memory recall response, but surprisingly these cells were not CD8⁺ T lymphocytes either. Nevertheless, the potential CD8-mediated mechanisms are currently under investigation.

It has been previously shown that targeting of the fixed immunogen, i*Ft*, to Fc γ R provided 20-50% protection in C57BL/6 mice against the more lethal *F. tularensis* strain, SchuS4 (Rawool et al., 2008). It would be of interest to investigate whether SchuS4 infection generates similar cellular immune responses, although the partial protection observed against this strain would account for more subtle differences among the immunized groups. For this reason, the current study focused primarily on *F. tularensis* LVS model, although the analysis of cell mediated mechanisms involved in SchuS4 protection is in the future scope of studies.

In summary, the present work advances our understanding of the *in vivo* protective mechanisms involved following Fc γ R targeting of a fixed bacterial immunogen. For the first time it is demonstrated that targeting of inactivated *F. tularensis* bacteria to Fc γ Rs reverses the potential detrimental effect of IL-10 during the early stages of *F. tularensis* infection. The anti-inflammatory cytokine IL-10 secreted by *F. tularensis* infected murine macrophages promotes the bacterial survival and replication by suppressing the synthesis of pro-inflammatory cytokines TNF- α , IL-12, IL-6 and IFN- γ . The immunization of mice with mAb-i*Ft* triggers activation of macrophages, shifts the

cytokine profile from anti-inflammatory towards pro-inflammatory and alters macrophage activation state from alternatively activated to classically activated macrophages. Intranasal immunization of mice with mAb-iFt IC increases the frequency of activated DCs as well as the generation of *F. tularensis*-specific, IFN- γ secreting, EM CD4⁺ T cells. Generation and maintenance of the EM T cell population is dependent upon IL-12 that is partially produced by activated lung DCs, but also systemically by other professional APCs. It is thought that the enhanced ability of the EM T cells to secrete IFN- γ is necessary for protection against *F. tularensis* challenge.

These findings support the hypothesis that targeting bacterial immunogens to Fc γ Rs on antigen presenting cells is an effective way to enhance innate immunity against intracellular pathogens during the early stages of infection. In addition, this is the first study that implicates Fc γ R in modulating DC and T cell responses in an infectious model *in vivo*, essentially bridging the innate and adaptive branch of the immune system. The enhanced cellular immune responses in the lungs and spleen of immunized mice observed in this model provide evidence for the generation of pathogen-specific mucosal and systemic immunity. Additional studies using the virulent type A *F. tularensis* SchuS4 strain will enhance our understanding of the pathogenesis and evasion mechanisms of *F. tularensis*. This targeting approach is highly applicable and promising as it fills a significant gap left by the paucity of FDA approved adjuvants and the lack of effective vaccines against mucosal pathogens.

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